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AWARD NUMBER AIBS 2691

TITLE: Arachidonate Metabolism in Breast Cancer Cultures: Identification of Antagonists/Agonists for Possible Intervention Strategies

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REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Final (1 Oct 94 - 30 Sep 98)

4. TITLE AND SUBTITLE
Arachidonate Metabolism in Breast Cancer Cultures: Identification of Antagonists/Agonists for Possible Intervention Strategies

5. FUNDING NUMBERS
AIBS 2691

6. AUTHOR(S)
Marti Jett, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington, DC 20307-5100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

There has been increasing evidence that high intake of dietary fats and obesity, in general, correlate with increased risk of breast cancer. The breast, as an apocrine gland shrouded in fat pads, may possess special regulatory mechanisms to protect the epithelial cells from stimulation in the rich mileu of the surroundings. Our hypothesis suggests that loss of protective mechanisms in breast tissue leaves breast epithelial cells vulnerable to unregulated stimulation. Our studies have addressed regulatory mechanisms in production of bioactive lipids. We have shown, using estrogen receptor + / cells, expression of various lipoxygenases (LO) and cyclooxygenase mRNA espression. Furthermore, inhibition of the LO pathway induced apoptosis. We identified that blocking other lipid metabolism enzymes caused cells to accumulate in Go/G1 phase of the cell cycle. We synthesized new classes of bioactive lipid targeting inhibitors. These water soluble, stable drugs effectively blocked proliferation in breast cancer cultures yet showed little toxicity in bone marrow cultures. Mice were given regimens for 3-10 weeks of the HPA-Na drug; necropsy revealed no toxicity other than hair loss at the site of drug administration. We have identified cross talk between MAP kinase pathway and arachidonic acid metabolism and have extensively characterized 8 different fatty acid binding proteins and find patterns unique to normal, localized or metastatic tumors. This finding has potential for diagnosis of breast cancer. These important mechanistic studies open up new avenues for selection of possible drug treatments and regimens to combat breast cancer.

14. SUBJECT TERMS
Breast Cancer
Eicosanoids, lipoxygenases, fatty acid binding proteins, bioactive lipids, hydroxyeicosatetraenoic ~~acid~~

15. NUMBER OF PAGES
128

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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(5) INTRODUCTION:

Studies with mice have shown a correlation between high levels of dietary fat and incidence of breast and prostate cancers (both of which are regulated by lipid hormones) {45}. Additionally, an association was described connecting obesity with increased risk of breast cancer. The breast, as an apocrine gland shrouded in fat pads, may possess special regulatory mechanisms to protect the epithelial cells from stimulation by products generated in the rich mileu of the surroundings. Our hypothesis addresses that issue suggesting that loss of protective regulatory mechanisms in breast tissue leaves breast epithelial cells vulnerable to unregulated stimulation. Lipid metabolism in the normal or cancerous breast is not well understood. A detailed discussion of arachidonate (AA [Appendix A contains Acronyms and Abbreviations]) metabolism, a lipid with far-reaching biological activities, is presented in Appendix B (pg 23) and a schematic summary is shown in Figure 1 (pg 28). This research proposal was initially prompted by the publication of epidemological data suggesting that people who took low dose aspirin daily for heart disease had a decreased incidence of colon cancer relative to a control group {26}. It was not clear if these individuals had also changed their food habits and life style or if other factors might also have contributed to the observed results. In our studies use of aspirin and other cyclooxygenase inhibitors to alter lipid metabolism in breast cancer cells, stimulated rather than inhibited proliferation in numerous breast cancer cell lines. Those experiments, however, do not address the issue of whether or not aspirin could serve to prevent the onset of malignant disease (as might be inferred from the studies in which low dose aspirin intake was correlated with diminished colon cancer). Furthermore, as we had seen previously with small cell lung cancer cells {43}, lipoxygenase, rather than cyclooxygenase inhibitors, blocked proliferation and induced apoptosis in breast cancer cells. These results can indicate various possibilities; for example, a) that certain blocked metabolites (or combinations of metabolites) are critical for proliferation, b) that diverted metabolism has caused the production of toxic metabolites, etc.

Therefore, in our studies we have evaluated the levels of approximately 30 arachidonic acid metabolites alone, in the presence of a growth factor +/- inhibitors which lead to apoptosis. Many of the arachidonate metabolites have been characterized as initiating cascades of other biologically active molecules such as cytokines, activation of kinases and calcium mobilization. These activators of cellular signals have even been shown to alter nuclear receptors that directly interact with DNA to have far-reaching effects on cellular functions {44}. In addition, fatty acid binding proteins (FABP) also have the potential to serve as signaling molecules and we

have found patterns of expression of FABP which appear to distinguish between normal, localized tumor and metastatic breast cancer. A detailed discussion of FABP's is presented in the Appendix C, pg 24.

We have used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects. Several of the 5-lipoxygenase inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis {1-3, 11}. We have used some of these in studies involving toxins, etc. and have demonstrated that these regulators of bioactive lipid generation block proliferation in a battery of cultured breast cancer cells including estrogen receptor positive and negative cultures and various malignant stages of cultures. Over the course of this study, we have begun to learn that generation of these bioactive lipids is unusually high in breast cancer cultures and blocking a single enzyme involved in these cascades of reactions, while a reasonably effective anti-proliferation strategy, can be improved. Therefore, we have directed many of our studies to examine the use of heteropolyanions that prevent many of the initial electron-transfers and free-radical intermediates essential for utilization of arachidonic acid.

In summary, we have made major advances in understanding the role of eicosanoids in breast cancer. These findings are presented in the attached manuscripts in the Appendices. The following summarized findings are a compilation of the major advances which are detailed in the attached 5 manuscripts. **1. Eicosanoids.** We have a) examined eicosanoids generated in response to growth stimulators, b) identified specific metabolites and showed that the metabolite, itself, increased proliferation, and c) delved further to identify expression levels of various eicosanoid-producing enzymes in both estrogen receptor (ER) +/- cells and in human breast tumor samples. **2. Drugs or inhibitors that target eicosanoid generation.** We have a) evaluated known inhibitors of specific pathways involved in lipid metabolism for anti-proliferative effects in breast cancer cultures of ER +/- cells, multidrug-resistant and metastatic cells and in xenograft mouse models of breast cancer, b) synthesized new families of inhibitors targeting lipid-metabolizing pathways and tested them for anti-proliferative activity, c) determined bone marrow toxicity of a host of inhibitors (aimed at lipid metabolism) to determine feasibility of continued investigation of these drugs for use in breast cancer (i.e. determine a therapeutic window), d) with selected inhibitors, performed pharmacokinetic studies in mice, e) determined cell cycle effects and examined apoptosis induced by inhibitors/drugs targeting lipid metabolism, and f) determined that the newly synthesized drugs did not induce multidrug-resistance. **3. Kinase activation and regulation of eicosanoid generation.** We have a) observed activation (phosphorylation) of Raf, MEK, MAP and 5-LO in breast cancer cells in response to growth factors, b) identified tyrosine phosphorylation of 5-LO within 1 minute of IGF-I stimulation, c) used specific inhibitors of these pathways to determine the effects on a host of phosphorylation reactions, and d) concluded that cross-talk between the eicosanoid-generating pathways and phosphorylation of regulatory kinases would provide new targets for therapeutic intervention strategies. **4. Lipid binding proteins (FABP), eicosanoids and regulation of cell proliferation.** We have identified patterns of various FABP typical of breast tumor vs normal cultures and project that specific patterns

are indicative of normal, localized tumors and metastatic breast cancer; this information serves as a foundation for screening body fluids as a routine test for breast cancer.

(6) BODY

MATERIALS:

Materials: Phenol red-free IMEM (improved minimal essential medium; BioFluids, Walkersville, MD); Complete culture fluid containing 7% fetal bovine serum, 1% MEM vitamins, appropriate growth factors (if required) and 1% antibiotics; all from BioFluids, Walkersville, MD; bioactive lipid standards and many specific inhibitors were purchased from BioMol Inc., Plymouth Meeting, PA or were gifts from Merck-Frosst, Pointe Clair, Montreal, Canada.

Cell cultures: Various breast cancer cultures including ER-positive ZR-75, T47D, and MCF-7 WT; ER-negative cultures include SKBR3, MB231, MCF-7 ADR^{10} and T47D-co cells. All were grown in phenol red-free IMEM to avoid problems with estrogen-like activities of phenol red. Cells were subcultured twice per week to prevent irreversible clumping. Cultures were discontinued after 15 subcultures and new cultures brought up from frozen stock. Cultures were tested weekly for estrogen receptor status. Normal breast epithelial cells were obtained from Dr. Vimla Band, New England Medical Center, and cultured according to instructions provided by Dr. Band.

METHODS:

1. **Establish quiescence in cultures of MCF-7 cells.** In the previous report, we showed that quiescence by serum depletion for 2 days produced cells that could respond to stimuli. This basic study showed that i) cells did not detach from the matrix, ii) cells had a decreased proliferation rate after 2 days culture in limited nutrients, and iii) cells recovered from the limited nutrient period and show proliferation both by thymidine incorporation and by microscopically determining the number of viable cells. The procedure can be summarized as follows:

    a) Day 1: Plate cells at a density which will permit them to remain in culture 1 week (10,000 /2 sq. cm. Plate in complete medium containing 7% fetal bovine serum and other usual additives. (Serum is necessary for the cells to attach to the plastic dishes).

    b) Day 3: Remove the fluid, gently wash the cultures with saline. Replace with serum-free medium containing NO additives.

    c) Day 4: Add agonist/antagonists to study generation of bioactive lipids, alterations in phosphorylation patterns, cross talk among regulatory pathways, etc.

2. **Description of proliferation assays to assess inhibition of cellular growth.** Cells were plated in 96 well cluster plates day 1, drug was added day 2, and on day 4 the experiment was ended by removal of culture fluid, placing the plate in the freezer (to disrupt cells). A lysis buffer was added to wells to further disrupt the cells and a ligand which emits fluorescence when bound to DNA was incubated for 10 min. Fluorescence was determined by using a Fluorescence detection plate reader.

3. **Detection of heteropoly anion (HPA) free-radical scavengers in tissues:** The mice administered HPA compounds were euthanized by CO2 and the organs examined and removed. Each organ was digested in 1M Nitric Acid, neutralized with 1 M NaOH and diluted. Tungsten was quantitatively determined by use of Atomic Absorption Spectral Analysis using ICP adaption. Syntheses of high oxidation state manganese-substituted heteropolyanions. We first isolated and characterized Mn^{IV} substituted Keggin polyanions ($[XW_{11}\ Mn^{IV}\ O_{40}\ ]^{n-}$, X = Si, B, and Zn) using X-ray diffraction, Extended X-ray absorption fine structure method, magnetic susceptibility, electrochemistry, and routine spectroscopic methods {35}. Di-manganese substituted γ-Keggin polytungstosilicates, γ-$[SiW_{10}\ Mn_2\ O_{40}\ ]^{m-}$, were synthesized{22-25, 29-34} . Oxidation of tetra-manganese substituted polyaions, $[P_2\ W_{18}\ Mn_4\ O_{68}\ \bullet 2H_2\ O]^{10-}$ game two mixed valent compounds were synthesized and characterized {28} as previously described {35-38}. Oxidation of alkylene with iodosobenzene (PhIO) was examined using above mono-, di-, and tetra manganese-substituted polyanions in the presence and absence of air. The oxidized products were identified by GC-Mass and comparison made of the retention time with authentic compounds.
4. **Arachidonate metabolism**: HPLC separation, identification and quantitation of ca. 30 arachidonate metabolites in a single run was devised by our laboratory {27}. We have established a pattern for determination of the kinetics of formation of even transient arachidonate metabolites in cell cultures +/- agonists or antagonists. The method can be adapted for the separation, and identification of radioactive metabolites from cell cultures, or for separating, identifiying and quantitating minuscule amounts (fMol & pMol) in samples from animals or tissues.
5. **Agonist stimulated arachidonate metabolism**: IGF-I stimulates proliferation quiescent MCF-7 cultures {43}. The objective of this proposal is to identify bioactive lipids which are being generated in response to growth factor stimulation in the presence and absence of specific arachidonate inhibitors or heteropolyanion treated MCF-7 cells. The MCF-7 cell cultures (an adherent cell line) will be plated in 4-well cluster plates and 24 hr later will be incubated overnight arachidonic acid (approximately 10 uCi/well). We have found this culture pattern to produce metabolites with sufficient radioactivity. The culture fluid will be removed, a saline wash performed, and buffer added (buffer designed for optimal phospholipase A2 activity). Control and agonist/antagonists will be added and the reaction stopped (acidified) at selected time periods from 15 sec through 2 hr (about 12 different time points for each series (control, agonist, etc.). We usually limit an experiment to approximately 50 wells. The cells will be scraped from the wells, briefly sonicated to insure cell disruption (some arachidonate metabolites may remain intracellular), and centrifuged at 20,000 x g to pellet all insoluble material. The internal standard is then added to the supernatant solution, the pH adjusted, arachidonate metabolites extracted and the samples prepared and run on reverse phase using a C-18 column for HPLC analysis. Platelet activating factor fraction will be separated away from arachidonate metabolites at the extraction step and will be assayed. Meanwhile, the pelleted material will be extracted by Bligh-Dyer procedure and the extracts applied to thin layer plates to run in solvent systems designed to resolve phospholipids or diglycerides/fatty acids. The latter techniques give interesting information when comparing

phospholipids present at the beginning of the experiment (samples under 2-3 minutes) with those present at the end of the experiment (>2 hr).

9. Toxicity testing in mice: HPA-SM was administered to Balb C, 6-8 week old mice using a 12 gauge needle inserted subcutaneously under the loose skin on the back of the mouse. HPA-NA (0.2 mg)was administered once weekly for 10 weeks, 0.5 for 7 weeks and 0.7 for 3 weeks. At the end of the time periods, the mice were sacrificed, extensive necropsy was performed and all organs were examined. No toxicity or abnormalities were observed. Plasma levels of drug were determined using atomic absorption spectrometry.

RESULTS AND DISCUSSION:

1. Manuscript #1 (submitted to *Journal of Clinical Investigation*), Appendix. In this study, 3 ER-positive and 3 ER-negative breast cancer cell lines were examined for mRNA expression for 5-, 12-, 15-LOX, FLAP, COX-1 and COX-2 transcripts. These analyses demonstrate that all cell lines tested uniformly express mRNA for 5-LO, FLAP, LTC-4 synthase and COX-1. COX-2 was expressed in 4/5 cell lines. LOX enzymes producing 12 & 15 modified eicosanoids were expressed in 3/6 and 5/6, cell lines respectively. Clinical specimens of malignant and normal breast tissue express mRNA for 5-LO. Exogenously added growth factors (transferrin and IGF-I) stimulated the synthesis of 5-HETE (a 5-LO product). Addition of 5-HETE to breast cancer cell cultures directly stimulated growth (>25%). Inhibition of 5-LO metabolism by selective antagonists resulted in growth reduction of the tumor cell lines tested. Inhibitors of the FLAP were most potent. In contrast, cyclooxygenase inhibitors at concentrations up to 100 uM were not toxic and were frequently stimulatory. Breast cancer cells exposed to 5-LO inhibitors demonstrated increased frequency of apoptosis; this is consistent with the mechanism for the anti-proliferative effect of the inhibitors being mediated via up regulation of apoptotic growth control.
2. Manuscript #2 (submitted to *Cancer Research*), Appendix. In this study, the attempt was to examine many more classes of inhibitors of eicosanoid metabolism. A pair of cell lines were chosen, the parent ER-positive MCF-7 and the multidrug resistant, ER-negative daughter clone MCF-7 ADR[10]. Figure 1 of the report (not the manuscript), shows a simplified schematic diagram of eicosanoid metabolism. The object of this study was to examine more fully the effects of blocking other key pathways in bioactive lipid generation. Among the classes of inhibitors examined were nonspecific LO inhibitors, a number of 5-LO or FLAP inhibitors, blockers of cytochome P-450 eicosanoid formation, PKC inhibitors and drugs which inhibit PAF formation. These findings show that targeting these lipid metabolism pathways is equally effective for drug-resistant and sensitive cells and is independent of ER status. We observed that the non-specific LO drugs caused accumulation of cells in Go/G1 phase and then led to apoptosis. In contrast, the 5-LO blocking drugs slowed the synchronized culture progression through the cell cycle, finally accumulating cells in G2/S prior to impending apoptosis. We further examined the most successful of these inhibitors in cultures of human bone marrow cells and found several of them to exhibit little toxicity

to the bone marrow cultures. The thorough examination of the bone marrow cultures included evaluation of various types of progenitor cells (table 1, report). Those data show that at effective concentrations of drugs, only CFU-E cells were affected. Even at lowest doses of drug, those cells were the most sensitive. However, the two lowest doses of drug did not reduce CFU-E below acceptable levels. These data, taken as a whole, suggest that these lipid metabolism pathways may be critical to survival of breast cancer cells and can be successfully targeted to control the rampant proliferation typical of breast cancer.

3. Manuscript #3 (under preparation for submission), Appendix. We synthesized a series of heteropolyanion drugs for targeting metabolism of breast cancer cells. Of these drugs, we selected 2 relatively non-toxic compounds for further studies. These drugs did not induced multidrug resistance, were equally effective in blocking proliferation and inducing apoptosis in drug resistant and sensitive cells and independent of ER-status. The drugs exhibit relative lack of toxicity in bone marrow cultures and in mice. These drugs have the capacity to be free-radical scavengers which means that they should block utilization of arachidonic acid and its subsequent metabolism. Preliminary data (this report, Fig 2), suggests a role in altering production of bioactive lipid intermediates. We are proceeding to evaluate the data for possible pursuit of IND approval. This family of drugs was included in a use patent for treatment of breast cancer.
4. Manuscript #4, Appendix (submitted to the *Journal of Biological Chemistry*). This manuscript attempts to put in perspective the mechanistic regulatory constraints involving mitogen-activated protein kinases (MAPK), stress activated protein kinases , signal transducers and activators of transcription (STAT), janus kinase (JAK). The data in this manuscript demonstrate that 5-LO pathway regulation impinges on the kinase activation cascades. These findings have been presented at an international meeting (International Congress of Biochemistry, San Francisco, August 97) and have generated a great deal of interest because of the expansion to our knowledge of the regulatory mechanisms which they have revealed.
5. Manuscript #5. In preparation-this will probably be divided into 2 manuscripts. Fatty Acid Binding Proteins (FABPs) in breast cancer. FABPs are named accouding to the tissue in which they were first identified. For example, Mammary-derived Growth Inhibitor (MDGI) has now been shown to be identical to what is known as Heart (H)-FABP. There have been indications that more than one type of FABP could appear in a tissue (eg. Kidneys contain Liver (L), Intestine (I) and Adipose (A) FABPs. Further, as we have discussed (pg 23) the MDGI disappears completely in breast cancer. We examined a wide variety of breast normal and cancer cells for a myriad of FABPs expressed. We have found, using RT-PCR primer pairs based on the cDNA for L- and I-FABPs (Figures 1a, b, page 24), 14- and 3-fold increases in tumor vs normal breast cells. In contrast, RT-PCR for A-FABP (Fig 1c) showed a 7-fold decrease in the expression level in cancer vs normal cells. A-FABP, heart-FABP and MDGI are similar in sequence and are found associated with normal cells. Differences were seen between T-47D and MCF-7 cells in response to I-FABP (Fig 1b). These results suggests that A-FABP may act as a tumor suppressors in breast cells similar to MDGI (H-FABP) in MCF-7 cells {46}. We are currently examining L- and I-FABPs and cell cycle patterns in MDGI sham and sense transfected MCF-7 cells and would predict that

MDGI sense-containing cells show suppression of I-FABP . Of the few FABPs tested so far, individually, each of these have been shown, in other cell systems, to correlate with the normal or tumor state. Our study is the first report to show decreases in the heart-type FABPs (A-FABP) concomitant with increases in mitosis promoting FABPs (I-FABP and L-FABP). We have synthesized a series of drugs, heteropolyanions (HPA), which are free-radical scavengers and target eicosanoid metabolism {18}. Use of HPA-Na, blocked cell cycle progression of MCF-7 cells and altered relative ratios of A- and I-FABP in cultures of tumor cells.

6. Additional information contained in graphs that are part of this report (in contrast being a part of one of the manuscripts).

Arachidonate metabolism in response to IGF-I in the presence and absence of various inhibitors of eicosanoid metabolism in T47D breast cancer cells. In all of the following graphs of AA metabolites, the "fold change" (Y-axis) was calculated by dividing the value for "control" cultures (no additions) into the value for the indicated regimens. Therefore, any fold-change other than "1", indicates a change relative to control. When inhibitors were used, they were added 30 min prior the fluid change which occurred immediately before addition of IGF-I. It is important to determine which of these metabolites rapidly appear (2 min) and which persist (30 min).

Figures 2-3, analyses of AA metabolites 2 min after agonist addition to cells shows several classes of metabolites elevated relative to control. This overview graph is used to identify, those metabolites showing the greatest changes relative to control values. IGF-I stimulated production of 5- and 15-HETE/HPETEs and their metabolites, methyl esters (ME) and δ-lactones (D LAC). IGF-I elevated most of these metabolites about 2-fold over control values. Quite striking was the elevation of most metabolites by MK591, the FLAP inhibitor alone, with values ranging from 12-fold increases in LTE4, to mostly 6-8 fold increases in prostanoids, LTB4, and 15-HPETE. Addition of IGF-I to cultures pre-incubated with MK591, brought all metabolite levels down to base levels except LTE4 and LTB4. Nordiguaretic acid (NDGA) alone showed no significant changes relative to control; NDGA + IGF-I showed an accumulation of 5-HPETE (the parent compound of the 5-LO pathway) and appeared to arrest further metabolism of AA to produce the incredibly potent 5- , and 15-LO bioactive lipids. The same analysis is indicated for the pair HPA-Na alone and HPA-Na + IGF-I. In that case, as with NDGA, 5-HPETE was elevated; the HPA-Na pair also forced the accumulation of AA, apparently preventing its utilization.

Figures 4-5, show analyses of AA metabolites 30 min after agonist addition to cells. IGF-I stimulated persistence of 5-LO products (LTC4 & 5-HPETE) and 15-HETE. By this time period, all three drugs +IGF-I stimulated accumulation of massive increased amounts of 15-HETE ME. The HPA-Na +IGF-I induced the accumulation of LTC4 about 24-fold relative to control values. In general, the less biologically active molecules were not present and parent or branchpoint molecules accumulated (see Fig 1). These data suggest that 5-, and 15-LO pathways are massively utilized by breast cancer cells. There

are few specific inhibitors available which target the 15-LO pathway. These data will possibly be included in one of the manuscripts described here as #5.

WORK ACCOMPLISHED COMPARED TO STATEMENT OF THE WORK :

Everything proposed was accomplished.

YEAR ONE

I. Characterize MCF-7 WT cell culture proliferation and/or inhibition in the presence of specific inhibitors of bioactive lipid generation including, arachidonate metabolism, such as aspirin/indomethacin, 5-lipoxygenase inhibitors, 15-lipoxygenase inhibitors or specific product inhibitors of 15-lipoxygenase metabolites. ***{Manuscripts 1,2,3}***

II. Establish the conditions for making MCF-7 cells quiescent. Determine that addition of growth factors or serum will induce proliferation in the quiescent cultures.. ***{Manuscript #4; Abstract A}***

III. Characterize MCF-7 WT cells with regard to proliferation in response to IGF-I and other appropriate growth factors.. ***{Manuscripts 1,2,3}***

YEAR TWO

I. Characterize the conditions for synchronizing cells in the G_0, resting cycle and then determine the time required for the cultures to advance to G_S. This will require the use of flow cytometry techniques to determine DNA with characteristics of specific cell cycle stages. Evaluate promising drugs/ inhibitors in this system to determine the phase in which they block proliferation. Use this as a basis for selecting drugs that have the potential to be most effective when used in combination.. ***{Manuscripts 1,2,4}***

II. Determine if breast cancer cells easily become resistant to selected promising inhibitors determined above and determine if they induce multidrug-resistance. These drugs which block bioactive lipid synthesis/ expression are designed to associate with cellular membranes rather than the cytosol and therefore we project that they will not easily be pumped out of multidrug-resistant cells; compare the effect of these drugs in blocking proliferation in multidrug-resistant cells as compared with wild-type cells.. ***{Abstract A}***

III. Characterize arachidonate metabolism in MCF-7 WT cells kinetically in samples +/- IGF-I and establish the effect of antagonists such as glucagon, on IGF-I stimulated cells. ***{Manuscripts 1,2 and Figures 2-5, this report}***

IV. Determine the arachidonate metabolites formed upon treating the cultures with aspirin, 5-lipoxygenase inhibitors and other appropriate inhibitors, depending on the outcome of the proliferation experiments.. ***{Manuscripts 1,2,3}***

YEAR THREE

I. With the knowledge gained from the previous two years of proliferation studies, apply information concerning growth factors, antagonists, and/or inhibitors to other breast cancer cell lines to see if common themes emerge. Test for synergy among various metabolic inhibitors.. ***{Manuscripts 1,2,3, Abstract B}***

II. Specific inhibitors identified as being unusually effective in blocking proliferation in breast cancer cultures will be evaluated for toxicity to normal human bone marrow (a) stroma cells and (b) to hemapoetic colony forming cells. Depending on the outcome of these studies, perform *in vitro* studies in mice to determine non-toxic dosing regimens.. ***{Manuscripts 2,3}***

III. Establish the relationship of JAK/STAT or RAS/RAF signaling cascades and their relationship with the generation of bioactive lipid metabolites; examine lipoxygenases for phosphorylation in response to IGF-I in quiescent cells. Determining the contribution of signaling kinases in quiescent cells in response to IGF-I and/or other growth factors. . ***{Manuscripts 4,5; Abstract C, D}***

IV. Closely examine the arachidonic acid metabolites and/or other biologically active metabolites such as cytokines, if indicated, which appear during the period of time between resting cells (G_0) and G_S. . ***{Manuscripts 1,2,3}***

V. Examine other breast cancer cell lines +/- growth factor(s), and inhibitors identified in the previous years to see if stimulation/inhibition of identified arachidonate metabolites can be confirmed as a general finding in breast cancer. . ***{Manuscripts 1,2,3}***

YEAR FOUR

I. Identify possible correlations between growth factor stimulation of specific arachidonate metabolites which are found to be common in breast cancer, identify the most effective inhibitor(s) and their possible combinations for potential intervention strategies for further investigations and possible clinical trials for treatment of breast cancer. . ***{Manuscripts 1,2,3, 5; Abstract E}***

II. Depending on the outcome of studies using specific inhibitors of arachidonate metabolism *in vitro*, examine animal models of breast cancer to see if the selected inhibitor(s) are effective *in vivo* in arresting breast cancer tumor development. . ***{Manuscripts 1,2,3}***

III. Summarize accumulated data

FUTURE DIRECTIONS AND GOALS:

1. Complete manuscripts in preparation and submit to peer reviewed journals
2. Continue to investigate HPA-NA drugs for treatment of breast cancer
3. Continue to define role of FABP in breast cancer.

PROBLEMS ENCOUNTERED:

No untoward problems were encountered this year. We would like to take this opportunity to say that the "Era of Hope" breast cancer review meeting was especially beneficial and urge the Command to organize such an event again.

ACCOMPLISHMENTS:

Patent filed: **M. Jett,** R. Das, R. Neill. Fatty acid binding proteins as diagnostic markers for breast and prostate cancer.

a) **FABP patterns for use in screening for breast cancer; diagnostic indicators of metastatic disease.**
b) **HPA drugs for use in treatment of breast cancer.** (We are exploring the possibility of IND evaluation for our newly synthesized HPA drugs such as HPA-NA or HPA-Sm).

MANUSCRIPTS:

1. Reduced proliferation of Breast Cancer by Interruption of the 5-Lipoxygenase Pathway of Arachidonic Acid Metabolism. Avis, Hong, Vos, Martinez, Moody, Jett and Mulshine. Submitted to J. Clin. Investigation.
2. Control of Growth of Human Breast Cancer Cells by manipulation of Arachidonate Metabolism. Zhang, Leung, Das, Mulshine, Jett. Submitted to Cancer Research.
3. Heteropolyanion Free-Radical Scavengers: Induction of Apoptosis in Human Breast Cancer Cells. Das, Zhang, Jett. Manuscript in preparation.

4. Cross Talk Between Map Kinase Pathway And Arachidonic Acid Pathway In The Signaling Cascade Of IGF-1 In Breast Cancer Cells. R. Das, N. Kodsi and M. Jett. Submission to J. Biol Chem.

5. Fatty Acid binding proteins in breast cancer. This will eventually be divided into 2 manuscripts, we expect; we are awaiting a massive amount of eicosanoid data from the statistician.

ABSTRACTS PUBLISHED WITH THE FUNDING OF THIS GRANT:

1. Das, R., Dhokalia, A., and M. Jett. Expression patterns of different Fatty Acid Binding Proteins in Breast Cancer Cells. Cancer Research (in press).
2. You, Yutong, X. Zhang, R. Das, B. Terry-Koroma and M. Jett. Cell Cycle Effects of Mammary Derived Growth Inhibitor in MDGI Gene Transfected Breast Cancer Cells (1997). Mol Biol of the Cell 8:88..
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(7) CONCLUSIONS

1. **Eicosanoids.** We have found that lipoxygenases, especially 5-LOX are crucial for breast cancer growth, that the expression of LOX and COX enzymes are abundant in breast cancer
2. **Drugs or inhibitors that target eicosanoid generation.** We have identified several drugs which block lipid metabolism that are universally effective anti-proliferative agents in a good variety of breast cancer

cell cultures and in mouse xenografts. We synthesized a family of drugs, HPA, that display a great deal of promise in terms of low toxicity in bone marrow assays and in mice, yet show excellent anti-breast cancer activity.

3. **Kinase activation and regulation of eicosanoid generation.** Characterized activation (phosphorylation) of Raf, MEK, MAP and 5-LO in breast cancer cells in response to growth factors, and identified tyrosine phosphorylation of 5-LO in breast cancer cells. Found that specific inhibitors of these pathways could provide new targets for therapeutic intervention strategies.
4. **Lipid binding proteins (FABP), eicosanoids and regulation of cell proliferation.** We have identified patterns of various FABP typical of breast tumor vs normal cultures and project that specific patterns are indicative of normal, localized tumors and metastatic breast cancer; this information serves as a foundation for screening body fluids as a routine test for breast cancer.

In summary, we have accomplished our goal of better understanding arachidonic acid metabolism in breast cancer. This is especially critical since fats and lipids have been correlated with increased risk of breast cancer. We have found that targeting lipid generating/metabolizing pathways provided effective anti-breast cancer responses. We have now designed and synthesized new families of drugs (HPA) which block these pathways. These drugs have been found to have low toxicity to bone marrow and in healthy mice while proving effective as anti-tumor agents. We have also clarified the role of regulatory enzymes involved in eicosanoid metabolism and have made stunning observations regarding the family of lipid binding proteins, FABPs. We have extensively characterized 8 different FABPs in cultures and human tumors and find patterns unique to normal, localized or metastatic tumors. We have proposed this latter finding as a potential routine diagnostic screening procedure using body fluids.

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(8) APPENDICES

Appendix A. Acronyms

Appendix B. Arachidonic acid metabolism: an overview

Appendix C. Fatty Acid Binding Proteins: an overview

Appendix D. Figure legends and Figures

Appendix E. Manuscripts and Abstracts

ABBREVIATIONS / GLOSSARY

AA = arachidonic acid or eicosatetraenoic acid, the parent eicosanoid compound.
LA= linoleic acid, a precursor molecule for biosynthesis of arachidonic acid
LO = lipoxygenase, one of two major series of enzymes metabolizing AA (See Fig 1).
HETE = hydroxyeicosatetraenoic acid (5-, 12-, 15-); The number associated with these metabolites indicate the carbon atom on which a hydroxyl has been added.
HPETE=hydroperoxyeicosatetraenoic acid; peroxy group added to AA at Carbon#.
LT=leukotrienes designated A,B,C,D,E4 (i.e., LTD4). Potent 5-LO metabolites with a wide range of biological activities.
Lipoxins= di- and tri-HETEs. Metabolites formed by hydroxylation of multiple carbon atoms of AA. Potent biological derivatives which stimulate protein kinase C
EA=eicosapentanoic acid; a synthetic eicosanoid which does not occur in nature. We have synthesized it for use it as an internal standard in our analyses of AA metabolism.
COX-I= The constitutive cyclooxygenase necessary for proper cellular function;
COX-2= The inducible cyclooxygenase, known to be responsible for pain and inflammation; may have other activities as yet undescribed
PLA2 = phospholipase A2, the enzyme which releases AA from phospholipids
FLAP = 5-lipoxygenase activating protein
PG= prostaglandins, such as PGE2= prostaglandin E2; PGF2, etc.
TBX=thromboxanes
EGF= epidermal growth factor;
IGF-I= insulin-like growth factor-I
JNK= Jun Kinase;
MAPK= Mitogen activated protein kinase
STAT=Signal transducers and activators of transcription
PPAR = perixosome proliferator-activated receptor
RXR = Retinoic X Receptor; RAR = Retinoic Acid Receptor
HPA's = heteropolyanions . A list of HPA's and an example of the structure are shown in manuscript #3.
FABP = fatty acid binding proteins; L-FABP= liver type; I-FABP= intestinal type; H- FABP = heart type; MDGI = mammary derived growth

inhibitor, a heart-type FABP; CRABP= Cellular Retinoic Acid Binding Protein
PKC=protein kinase C

APPENDIX B

Arachidonic Acid Metabolism.

Arachidonic acid is released from the Sn-2 position of phospholipids by the family of enzymes phospholipase A2 (PLA2). There are small soluble secreted PLA2's, and PLA2's which are located within the nucleus. The newly released arachidonic acid is, then, the substrate for numerous enzymes including lipoxygenases (LO); a 5-lipoxygenase activating protein (FLAP) is thought to form a complex with 5-LO and anchor it to the inner aspect of the nuclear membrane producing numerous bioactive lipids which initiate the cascades of mediator release. A simplified chart of arachidonic acid metabolism is shown in Fig. 1. The two major classes of enzymes responsible for arachidonic acid metabolism are the cyclooxygenases (COX) and lipoxygenases (LO). Cyclooxygenases produce the prostanoids and thromboxanes. The best known of these is probably prostaglandin E2, the compound involved in pain and inflammation. Other prostanoids have been implicated in cell proliferation. A multidrug-resistant breast cancer cell line{4,5}, MCF-7 ADR^{10}, has greatly increased levels of prostacyclin H synthetase {28}. Lipoxygenases alter arachidonic acid by first adding a hydroperoxy group (through a free-radical mechanism) at the so named (5-, 12- 15-, etc.) carbon position, and the resulting compounds are named hydroperoxyeicosatetraenoic acids (HPETE). The second generation compounds are hydroxyeicosatetraenoic acids (HETE's). The lipoxygenases are named for the carbon position that they modify; the 5, 12, and 15 being the most common. The 12- and 15-HETE's have been shown to stimulate cell proliferation in low concentrations {1-3}, however, they become quite toxic at higher concentrations. The 5-lipoxygenase (LO) pathway has been studied extensively because of its involvement in acute allergy symptoms, shock, adult respiratory distress syndrome{2}. We have identified a 5-LO metabolite, 5-HETE, as a possible growth (co)factor in small cell lung carcinoma and now, as will be described, have implicated it in breast cancer. This compound is also converted to lipoxins, metabolites characterized to stimulate protein kinase C. Lipoxins are abundantly produced in response to growth factors in the tumor cell systems, which we are studying. In addition, specifically hydroxylated linoleic acid has been shown to occur upon stimulation of 3T3 cells with EGF {8}. It is unclear if the same lipoxygenase utilize both arachidonic and linoleic acids.

APPENDIX C

Fatty acid binding protein involvement in utilization/trafficking of mitogenic lipids

Intracellular utilization of bioactive lipids is a critical component in the process by which these molecules continuously stimulate proliferation through nuclear receptor interactions. Transport/ utilization are proposed to be mediated by the cytoplasmic proteins known as fatty acid binding proteins (FABPs) [1]. These proteins may also be important in regulating intracellular fatty acid concentrations. Such crucial participation in the trafficking and availability of fatty acids implicate FABPs as critical links in the mechanistic chain connecting dietary fat with cancer.

FABPs are found in abundance in a variety of tissues. The members of this broad multigene family currently consist of at least seven types whose amino acid sequences were determined from purified proteins or cDNA nucleotide sequences from tissue RNA [2-8]. Initially, an FABP was named for the tissue from which it was isolated and includes: 1) adipocyte (A-FABP), 2) heart or muscle (H-FABP), 3) brain (B-FABP), 4) epidermis or psoriasis-associated (E-FABP), 5) liver (L-FABP), 6) intestine (I-FABP), and 7) myelin or P2 (P2-FABP). A frequently studied FABP from bovine mammary gland, designated MDGI (mammary-derived growth inhibitor), thought to be a distinct type in itself, was later identified as H-FABP [9, 10]. Expression of each FABP type is not necessarily limited to the tissue from which it was originally isolated. In some tissues FABP expression is developmentally regulated and different types may be expressed in different regions of an organ.

The properties common to FABPs include their intracellular abundance, their small size (a molecular weight range of 14-16 kDa and an average of 132 amino acids), their sequence relatedness and three-dimensional structure and their ability to bind a variety of lipids. As a group A-FABP, H-FABP, B-FABP, and E-FABP in humans share between 50-65% protein sequence homology and contain a tyrosine near residue 20 that can be phosphorylated. These four FABPs share only 20-25% homology with L-FABP or I-FABP which do not have the tyrosine. L-FABP is distinguished by its lack of the amino acid tryptophan.

Certain FABPs have been reported to have differential effects on cell growth when cDNA clones have been transfected into cells. Transfection of L-FABP into hepatoma cells increased proliferation [1, 11, 12]. In contrast, MDGI (H-FABP) appears only in normal and not tumor mammary cells [13-14]; transfection of a cDNA clone of MDGI into breast cancer cells or mouse mammary epithelial cells resulted in loss of tumorigenicity [15]. FABPs are known to bind many different groups of fatty acids and their derivatives, and other bioactive lipids [reviewed in [16]. L-FABP exhibits different lipid binding characteristics from that of A-FABP or H-FABP. L-FABP transfected into rat hepatoma cells also mediates cell induction by carcinogenic peroxisome proliferators [17], and involves alteration of eicosanoid metabolism (Panandiker et al [addendum J-B]). FABP causes a net diffusion of fatty acids from to intracellular membrane compartments [18, 19].

Changes in expression of FABPs have been reported for bladder cancer. Psoriasis-associated FABP (E-FABP) was noted to increase in level with increase in differentiation of bladder squamous cell carcinomas [20]. Although FABPs are intracellular proteins, H-FABP has been detected in elevated levels in plasma and urine of patients suffering from myocardial infarction [21-22], whereas psoriasis-associated FABP (E-FABP) was among a number of marker proteins detected in the urine of bladder cancer patients [23]. In addition, loss of adipocyte-FABP (A-FABP) was reported with progression of human bladder transitional cell carcinomas [24]. The presence of A-FABP correlated with the grade and stage of the disease. The A-FABP protein was present in high levels in grade I and II TCCs whereas grade III had 37% reduction and grade IV had no A-FABP expression. A-FABP may act as a growth inhibitor similar to the MDGI (H-FABP) protein in breast cancer and loss of A-FABP expression may serve as a prognostic marker for aggressive bladder cancer. Our preliminary data are the only studies of the different levels of FABPs in normal and cancerous breast cells.

MDGI has been regarded as being associated with the "normal" breast since it is singularly absent in the breast cancer cells/tissues examined and when added exogenously, blocks growth of breast cancer cells in vitro. However, little note has been taken of the fact that it is absent in "normal" breast tissue, but is abundantly produced during lactation (specialized normal). We suggest that the role of MDGI, in that specialized state, is to bind, and thus, protect the epithelial cells from the incredibly rich mileu of bioactive lipids and other factors that bath the cells during lactation. We postulated that other FABPs were playing a role in normal/tumor breast cells; we now have preliminary data implicating an altered balance of FABPs in normal vs tumor breast cells.

Current studies in our laboratory regarding fatty acid binding proteins in breast cancer: We have found, using RT-PCR primer pairs based on the cDNA for L- and I-FABPs (Figures 1b, a), 14- and 3-fold increases

in tumor vs normal breast cells. In contrast, RT-PCR for A-FABP (Fig 1c) showed a 7-fold decrease in the expression level in cancer vs normal cells. A-FABP, heart-FABP and MDGI are similar in sequence and are found associated with normal cells. Differences were seen between T-47D and MCF-7 cells in response to L-FABP (Fig 1b). These results suggests that A-FABP may act as a tumor suppressors in breast cells similar to MDGI (H-FABP) in MCF-7 cells [15]. We are currently examining L- and I-FABPs and cell cycle patterns [addendum J-Fig. D] in MDGI sham and sense transfected MCF-7 cells and would predict that MDGI sense-containing cells show suppression of I-FABP . Of the few FABPs tested so far, individually, each of these have been shown, in other cell systems, to correlate with the normal or tumor state. Our study is the first report to show decreases in the heart-type FABPs (A-FABP) concomitant with increases in mitosis promoting FABPs (I-FABP and L-FABP). We have synthesized a series of drugs, heteropolyanions (HPA), which are free-radical scavengers and target eicosanoid metabolism [1]. Use of HPA-Na, blocked cell cycle progression of MCF-7 cells and altered relative ratios of A- and I-FABP in cultures of tumor cells [addendum B-Figures A-D & F].

Expression of Various Fatty Acid Binding Proteins in Breast Cells.

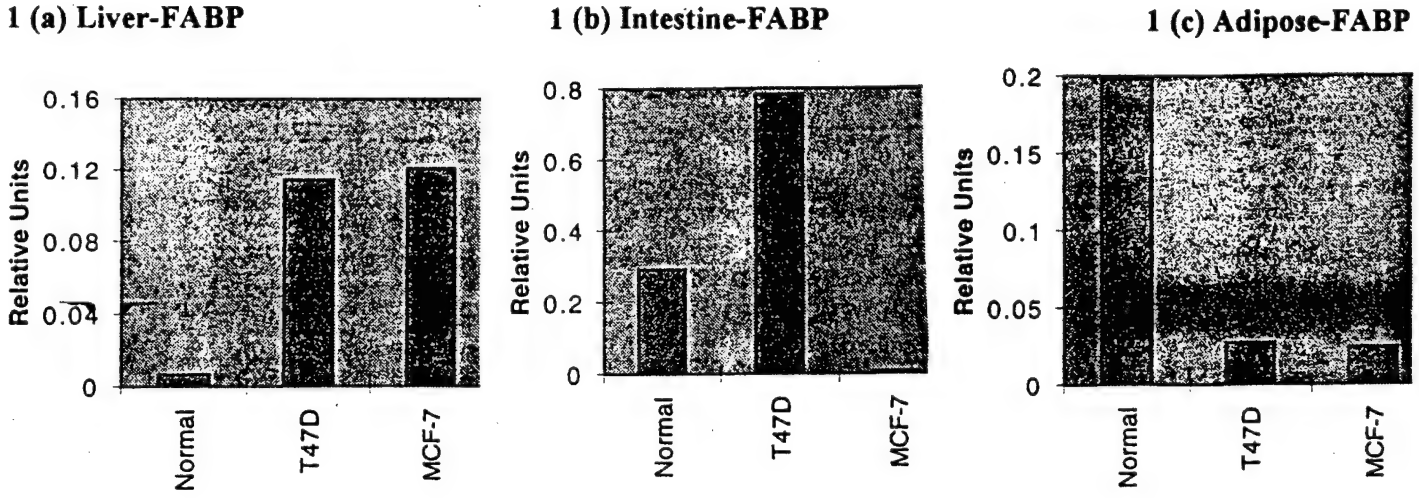

Figure 1. RNA was extracted from breast normal and tumor cultured cells. PCR primers for Liver- (a), Intestine-(b) and Adipose-FABPs (c) were used to determine the levels of expression of the specific FABP.

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22. Sohmiya, K., *et al.*, *Plasma and urinary heart-type cytoplasmic fatty acid-binding protein in coronary occlusion and reperfusion induced myocardial injury model.* J Mol Cell Cardiol, 1993. **25**(12): p. 1413-26.
23. Van Nieuwenhoven, F.A., *et al.*, *Discrimination between myocardial and skeletal muscle injury by assessment of the plasma ratio of myoglobin over fatty acid-binding protein.* Circulation, 1995. **92**(10): p. 2848-54.
24. Wodzig, K.W., *et al.*, *Estimation of myocardial infarct size from plasma myoglobin or fatty acid-binding protein. Influence of renal function.* Eur J Clin Chem Clin Biochem, 1997. **35**(3): p. 191-8.

LEGENDS TO FIGURES

Figure 1. A schematic diagram showing some of the major bioactive lipid metabolites of arachidonic acid. Pathway A indicates non-specific lipoxygenases; B, 5-lipoxygenases, C, peptidoleukotrienes; and D, cyclo-oxygenases.

Figure 2-5. Arachidonic acid metabolites produced in response to IGF-I in the presence and absence of various drugs that target lipid metabolism. In all of the following graphs of AA metabolites, the "fold change" (Y-axis) was calculated by dividing the value for "control" cultures (no additions) into the value for the indicated regimens. Therefore, any fold-change other than "1", indicates a change relative to control. When inhibitors were used, they were added 30 min prior the fluid change which occurred immediately before addition of IGF-I.

Figures 2-3. Analysis at 2 min post IGF-I for "5-LO products" (Fig. 2) and "Prostanoids & 12-, 15-LO products (Fig. 3).

Figures 4-5. Analysis at 30 min post IGF-I for "5-LO products" (Fig. 4) and "Prostanoids & 12-, 15-LO products (Fig. 5).

Figure 1

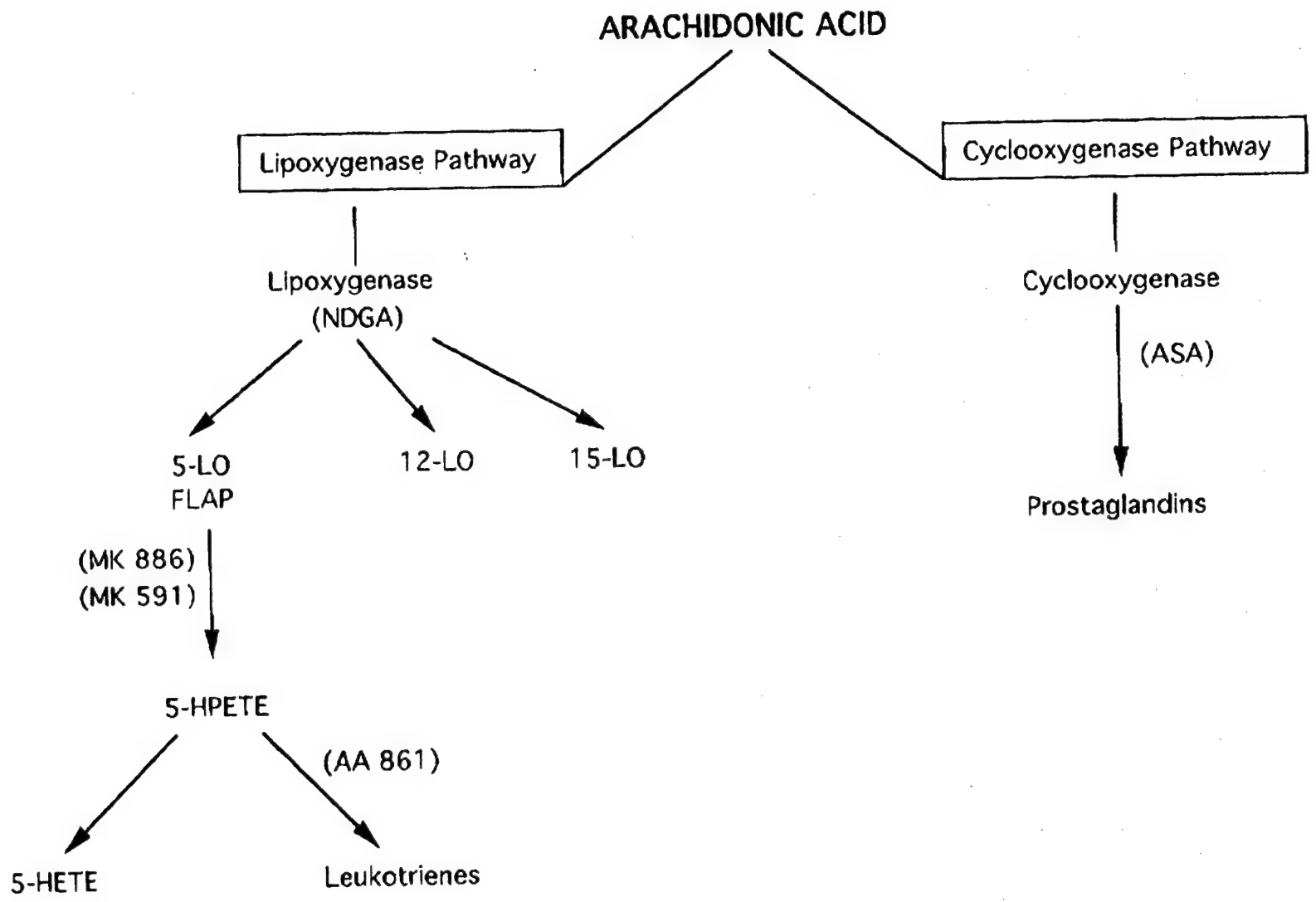

ARACHIDONIC ACID
Lipoxygenase Pathway
Cyclooxygenase Pathway
Lipoxygenase
(NDGA)
Cyclooxygenase
(ASA)
5-LO
FLAP
12-LO
15-LO
Prostaglandins
(MK 886)
(MK 591)
5-HPETE
(AA 861)
5-HETE
Leukotrienes

Figure 2

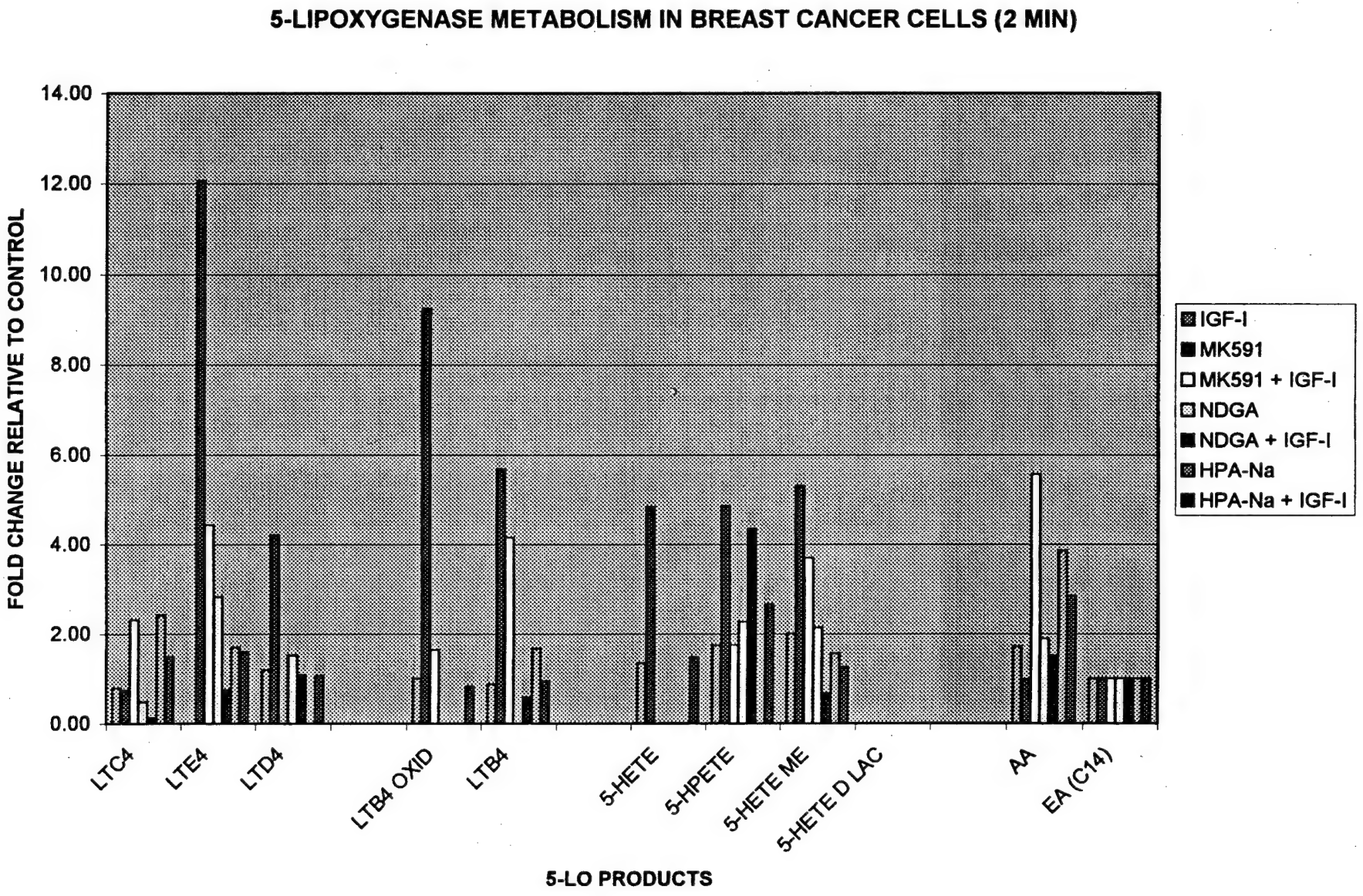

5-LIPOXYGENASE METABOLISM IN BREAST CANCER CELLS (2 MIN)
FOLD CHANGE RELATIVE TO CONTROL
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
LTC4
LTE4
LTD4
LTB4 OXID
LTB4
5-HETE
5-HPETE
5-HETE ME
5-HETE D LAC
AA
EA (C14)
5-LO PRODUCTS
IGF-I
MK591
MK591 + IGF-I
NDGA
NDGA + IGF-I
HPA-Na
HPA-Na + IGF-I

Figure 3

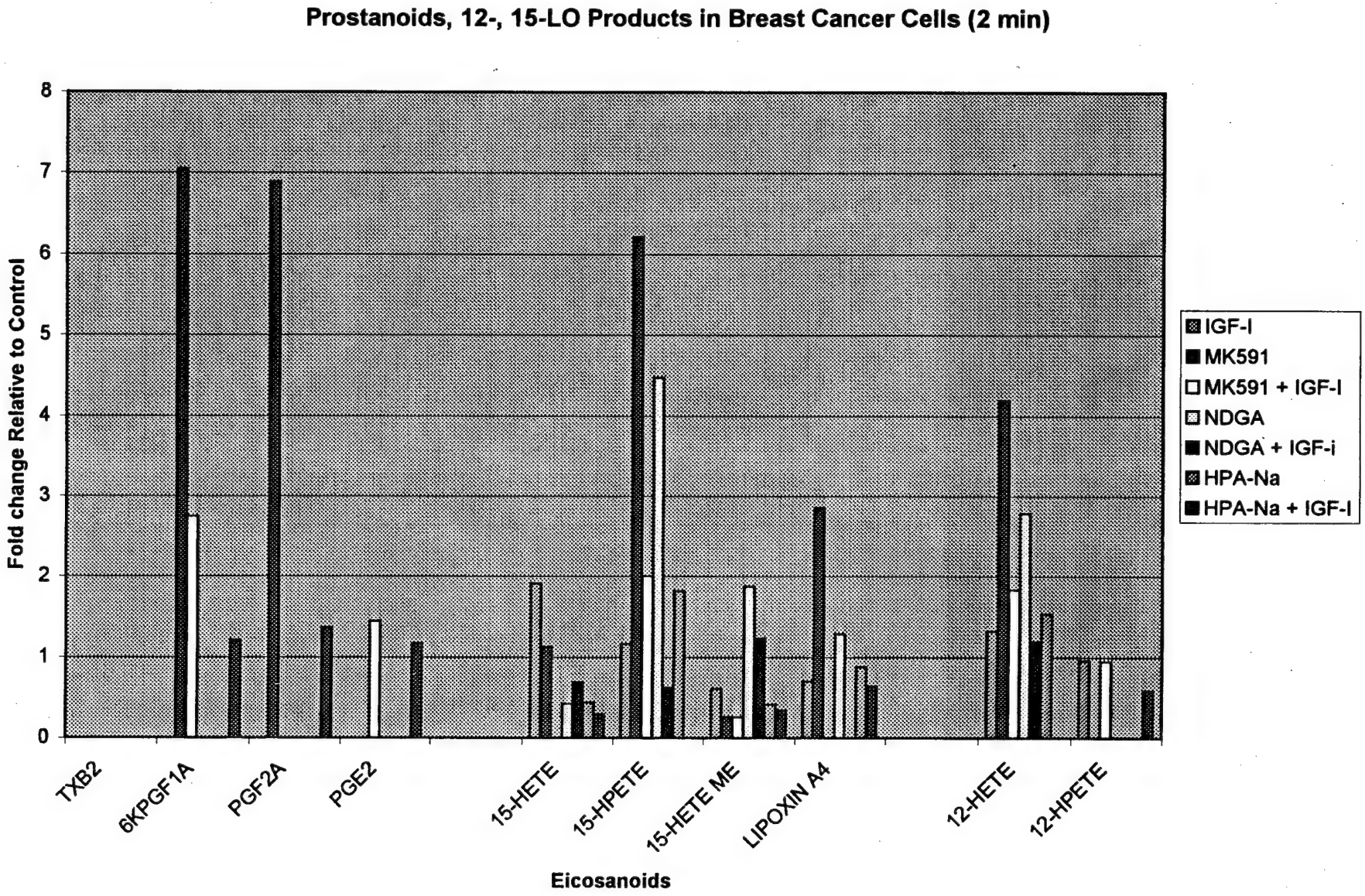

Prostanoids, 12-, 15-LO Products in Breast Cancer Cells (2 min)
Fold change Relative to Control
0
1
2
3
4
5
6
7
8
TXB2
6KPGF1A
PGF2A
PGE2
15-HETE
15-HPETE
15-HETE ME
LIPOXIN A4
12-HETE
12-HPETE
Eicosanoids
IGF-I
MK591
MK591 + IGF-I
NDGA
NDGA + IGF-i
HPA-Na
HPA-Na + IGF-I

Figure 4

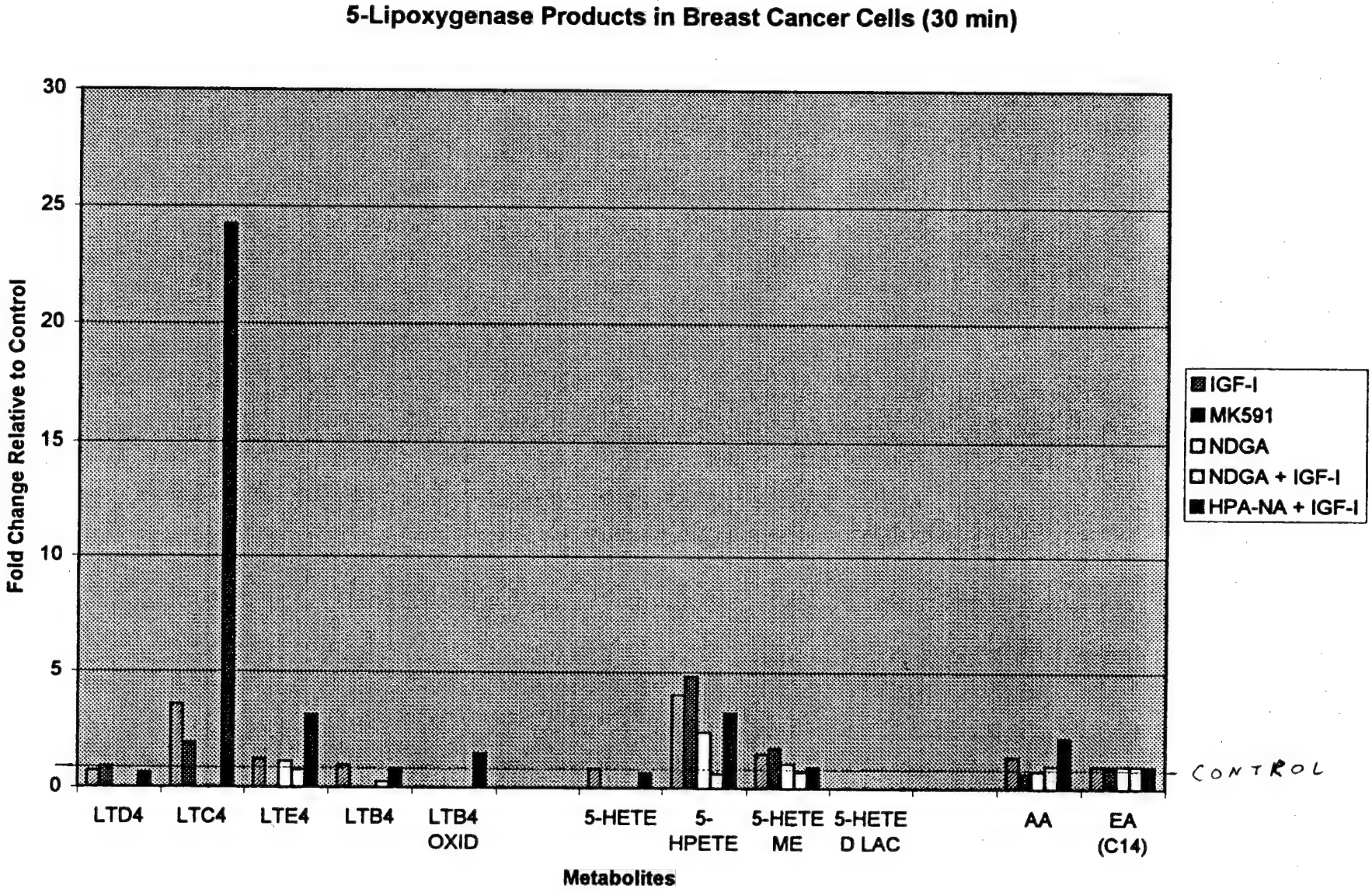
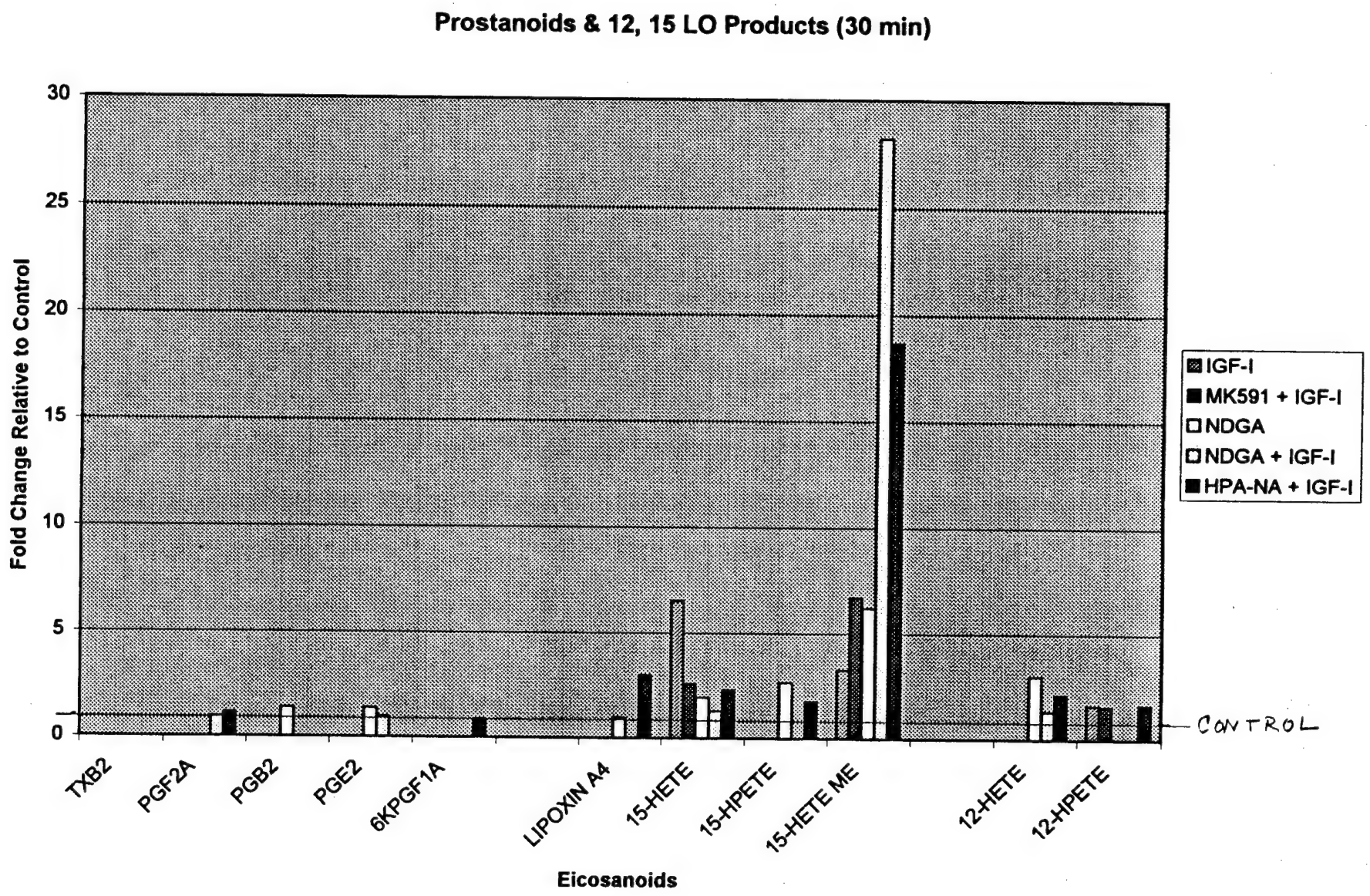

5-Lipoxygenase Products in Breast Cancer Cells (30 min)
Fold Change Relative to Control
30
25
20
15
10
5
0
IGF-I
MK591
NDGA
NDGA + IGF-I
HPA-NA + IGF-I
CONTROL
LTD4
LTC4
LTE4
LTB4
LTB4 OXID
5-HETE
5-HPETE
5-HETE ME
5-HETE D LAC
AA
EA (C14)
Metabolites

Figure 5


Prostanoids & 12, 15 LO Products (30 min)
Fold Change Relative to Control
30
25
20
15
10
5
0
IGF-I
MK591 + IGF-I
NDGA
NDGA + IGF-I
HPA-NA + IGF-I
CONTROL
TXB2
PGF2A
PGB2
PGE2
6KPGF1A
LIPOXIN A4
15-HETE
15-HPETE
15-HETE ME
12-HETE
12-HPETE
Eicosanoids

E. ATTACHED MANUSCRIPTS

SELECTED ABSTRACTS PUBLISHED WITH THE FUNDING OF THIS GRANT:

A. Y. Wang, X. Zhang, J. Weitz and M. Jett (1996). Heteropolyanions do not Induce P-Glycoprotein associated with multidrug Resistance in Human Breast Cancer Cells. FASEB Journal 10:A1145.
B. Bioactive Lipids in Breast Cancer. XiaoYan Zhang, Rina Das and **Marti Jett** (1997). Proceedings, Era of Hope Breast Cancer Meeting, Vol III: 1009-1010.
C. Das, R., Kodsi, N. and Jett, M. (1997) Cross talk between the MAP kinase and the arachidonic acid pathway in signal transduction of growth factor in breast cancer cells. FASEB Journal 10: 2852.
D. You, Yutong, XY. Zhang, R. Das, Terry-Koroma and M. Jett. Cell Cycle Effects of Mammary Derived Growth Inhibitor in MDGI Gene Transfected Breast Cancer Cells (1997). Mol Biol of the Cell 8:88.
E. Das, R., Dhokalia, A., and M. Jett. Expression patterns of different Fatty Acid Binding Proteins in Breast Cancer Cells. Cancer Research (in press).

Reduced Proliferation of Breast Cancer by Interruption of the 5-Lipoxygenase Pathway of Arachidonic Acid Metabolism.

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[3]The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense (Para.4-3) AR 360-5

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Abstract

Arachidonic acid metabolism has an important function in promoting the growth of cancer cells. Previously, we demonstrated that the lipoxygenase pathway of arachidonic acid metabolism is important in the growth regulation of lung cancer, we now report a similar role for this pathway in breast cancer. In this study, six breast cancer cell lines were analyzed for mRNA expression for lipoxygenase and cyclooxygenase transcripts. These analyses demonstrate that all cell lines tested uniformly express mRNA for 5-lipoxygenase, 5-lipoxygenase activating protein, LTC-4 synthase, and cyclooxygenase-1. In contrast cyclooxygenase-2 was expressed in 4/6 cell lines, 12, and 15-lipoxygenase were expressed in 3/6, and 5/6, cell lines respectively. We also show evidence that clinical specimens of malignant and normal breast tissue express mRNA for 5-lipoxygenase. Two exogenously added autocrine growth factors, insulin-like growth factor-1 and transferrin, induced the biosynthesis of the 5-lipoxygenase metabolite 5-HETE. Using a proliferation growth assay, our results demonstrate that 5-HETE can directly stimulate growth (>25% over control at 0.01 ug/ml) *in vitro*. Furthermore, inhibition of lipoxygenase metabolism by selective antagonists at 1-10 uM concentrations resulted in significant, reproducible growth reduction of 20-90% *in vitro* of the tumor cell lines tested. Inhibitors of the 5-lipoxygenase activating protein were most potent. In contrast, cyclooxygenase inhibitors at concentrations <100 uM had no significant effect. Breast cancer cells exposed to lipoxygenase inhibitors

demonstrate increased frequency of apoptosis, which is consistent with the mechanism for the anti-proliferative effect of the inhibitors being mediated via up regulation of apoptotic growth control. These results suggest that inhibitors of the lipoxygenase pathway may provide a new pharmacological method to reduce the growth of breast cancer cells.

Table 1. mRNA Expression in Breast Cancer Cell Lines

Cell line	5-LO	FLAP	LTC4	12-LO	15-LO	COX-1	COX-2
ER-Positive							
MCF7 (WT)	+	+	+	+	+	+	-
ZR75	+	+	+	+	+	+	-
T47D	+	+	+	-	+	+	+
ER-Negative							
SKBR3	+	+	+	-	-	+	+
MB231	+	+	+	-	+	+	+
MCF7-adr	+	+	+	+	+	+	+

mRNA expression of *5-LO, FLAP, LTC4, 12-LO, 15-LO, COX-1* , *COX-2* , as analyzed by RT-PCR and southern blot analysis in six different breast cancer cell lines. The experiment was repeated twice.

Table 2: Effect of Exogenous 5-HETE on Growth Proliferation

Cell Lines	uM 5-HETE	% Maximum Growth
ER-Positive		
MCF-7 (WT)	0.1	50
ZR-75	0.1	40
T47D	0.01	25
ER-Negative		
SKBR3	0.1	<10*
MB231	0.02	30
MCF-7 (ADR)	ND	

Percent maximum growth was calculated from the optical density value from a minimum of 6 replicates from at least three different experiments per cell line. Percent growth inhibition compared to vehicle control was significantly different ($p<0.05$) except where specified. (* indicates no significant difference in growth compared to control). ND: not done

Table 3. Growth Inhibition in Breast Cancer Cell Lines in the Presence of Specific Inhibitors of the AA Metabolic Pathways

Cell Lines	NDGA	AA861	MK886	MK591	ASA
ER-Positive					
MCF7 (WT)	74 ± 13	58 ±12	88 ± 7	100 ± 0	<10*
ZR-75	57 ± 28	33 ± 6	92 ± 10	95 ± 5	<10*
T47D	83 ± 6	<10*	53 ± 6	60 ± 0	<10*
ER-Negative					
SKBR3	73 ± 12	<10*	77 ± 15	90 ± 0	<10*
MB231	65 ± 21	<10*	90 ± 17	93 ± 11	<10*
MCF7-adr	75 ± 9	60 ± 23	72 ± 19	100 ± 0	<10*

The mean growth inhibition ± s.d. of a minimum of 3 experiments is presented, and drug concentrations used were 5 uM for NDGA, MK 886, and MK591, 10 uM for AA861, and 100 uM for ASA. For each compound the drug concentration that showed the greatest mean inhibition is shown. All values were determined by assessment of % growth inhibition calculated from the optical density value, with a minimum of 6 replicates from at least three different experiments per cell line. Percent growth inhibition compared to vehicle control was significantly different ($p<0.05$) except where specified. (* indicates no significant difference in growth compared to control).

Table 4. Effect of LO Inhibitors on MCF-7 Xenografts

Compound	Tumor volume mm^3
PBS	2394 ± 591
MK591	1318 ± 342 ($p>0.05$; NS)
NDGA	765 ± 142 ($p<0.02$)

Nude mice were injected with MCF-7 (WT) cells, and xenografts formed after one week. The mice were administered daily MK 591, NDGA or PBS for weeks 2-4, and the tumors were measured biweekly. The mean value ± S.E. of 5 determinations is indicated.

Figure legends:

Figure 1: Schematic outline of AA metabolism. The released arachidonic acid is metabolized either by cyclooxygenase pathway (right), or the lipoxygenase pathway (left). The proposed site of action for the inhibitors used in the study is shown in parenthesis.

Figure 2. Southern blot analysis showing mRNA expression of 5-LO and its associated activating protein (FLAP) in 6 different breast cancer cell lines (MB-231, MCF-7 WT, MCF-7 ADR, ZR-75, T47D, and SKBR-3) using RT-PCR.

Figure 3: Detection of 5-LO mRNA by in situ RT-PCR in paraffin sections taken from morphologically normal breast tissue (A, B), and malignant breast tumor tissue (C, D).. Negative controls (B, D) were performed by substituting the primers by water in the PCR mixture. Magnification: A, B x67; C, D x333.

Figure 4. Production of endogenous 5-HETE after exposure to two growth factors. The cell lines used were MCF-7 WT; ZR-75; T47 D; and SKBR3. The result is presented as pMOL/0.1 ml 5-HETE and its derivatives released after exposure to 10 ug/ml TF (B), and 5ug/ml IGF-I (A), using a RIA kit. A: Baseline values. Error bars indicate SEM, and all 5-HETE values were statistically significant from baseline values ($p<0.05$).

Figure 5. Effect of exogenous addition of 5-HETE on the proliferation of breast cancer cell line ZR-75, in the presence of medium containing insulin and selenium. A representative experiment is shown, and the result is expressed as mean optical density ± standard deviation of a six data points. Asterisks indicates significant stimulation as compared to vehicle control ($p<0.05$). Maximal stimulation was observed at 0.05 mg/ml 5-HETE.

Figure 6. Effect of inhibitors of AA metabolism on breast cancer cell lines. Representative experiments on breast cancer cell lines ZR-75 (A), MCF-7 WT (B), and SKBR-3 (C) is shown for each compound. The inhibitors are NDGA (open squares), AA861(closed squares) and MK886 (closed circles). The experimental conditions were as outlined in Methods, and the results are expressed as % growth ± standard deviation, with no drug added serving as control and expressed as 100% growth. Asterisks indicates significant growth inhibition versus control ($p<0.05$). Dose concentrations used were 0.5, 1, 5 and 10 mM with all three compounds.

Figure 7: Effect of NDGA on the in vitro proliferation after exposure to IGF-I.

Figure 8: *In vitro* apoptosis analysis after treatment with two inhibitors. Results are presented as percent apoptotic cells (A) in four breast cancer cell lines (MB-231, SKBR-3, T47 D, and ZR-75). Control (filled bars),

treatment with 2 uM MK 591 (shadowed bars), and treatment with 2 uM NDGA (open bars). Morphological changes in breast cancer cell line MB-231 treated with 2 uM NDGA (C), or 2 uM MK 591 (D) as compared to control (B). Magnification: x 290.

Figure 9: Effect of administration of two inhibitors on apoptosis *in vivo*. Histological sections were stained for apoptosis in heterotransplanted tumors of breast cancer cell line MCF-7 (WT). Data represent the average ± standard deviation in ten microscopic fields counted. The difference between the treated samples and control samples was statistically significant ($p<0.05$).

Acknowledgment:

We thank Dr. G. Allred for the kind gift of the pathological breast specimens, and Dr. B. Murphy for helpful discussions. We also thank Merck Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada), for the kind gift of FLAP inhibitor MK-591. In addition, we are grateful to Amy Guzzone, and Valerie McKey for excellent technical assistance. Partial support for this work was provided by the Leila Y. and Harold G. Mathers Charitable Trust.

Abbreviations used in this paper:

AA, arachidonic acid; LO, lipoxygenase; COX, cyclooxygenase; FLAP, 5-lipoxygenase activating protein; IGF-R, insulin-like growth factor receptor; ER, estrogen receptor; RT-PCR, reverse transcriptase polymerase chain reaction; RIA, radioimmunoassay; ASA, acetylsalicylic acid; NDGA, nordihydroguaiaretic acid;

Figure 1

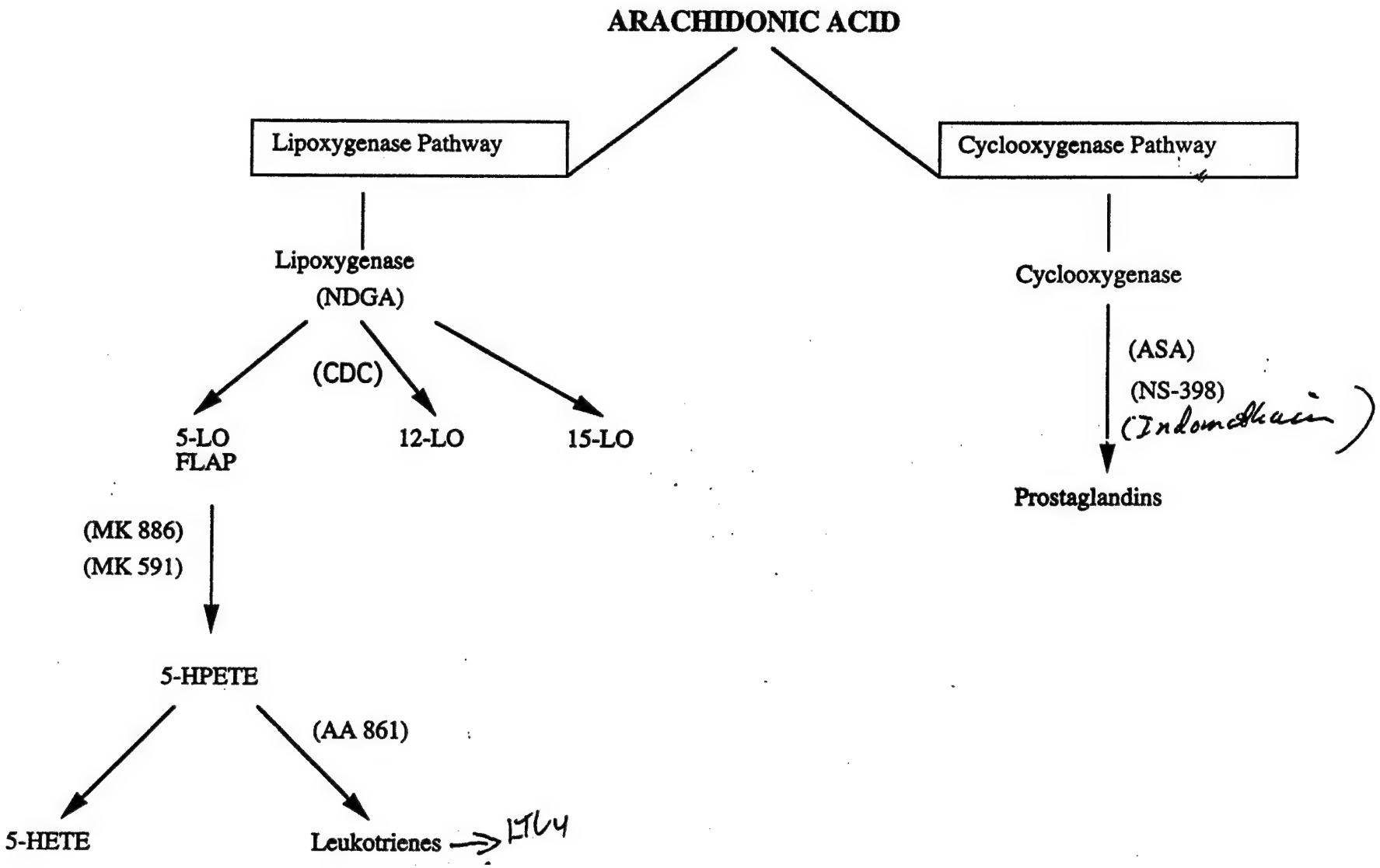

ARACHIDONIC ACID
Lipoxygenase Pathway
Cyclooxygenase Pathway
Lipoxygenase
(NDGA)
(CDC)
5-LO
FLAP
12-LO
15-LO
(MK 886)
(MK 591)
5-HPETE
(AA 861)
5-HETE
Leukotrienes
LTC4
Cyclooxygenase
(ASA)
(NS-398)
(Indomethacin)
Prostaglandins

Figure 2

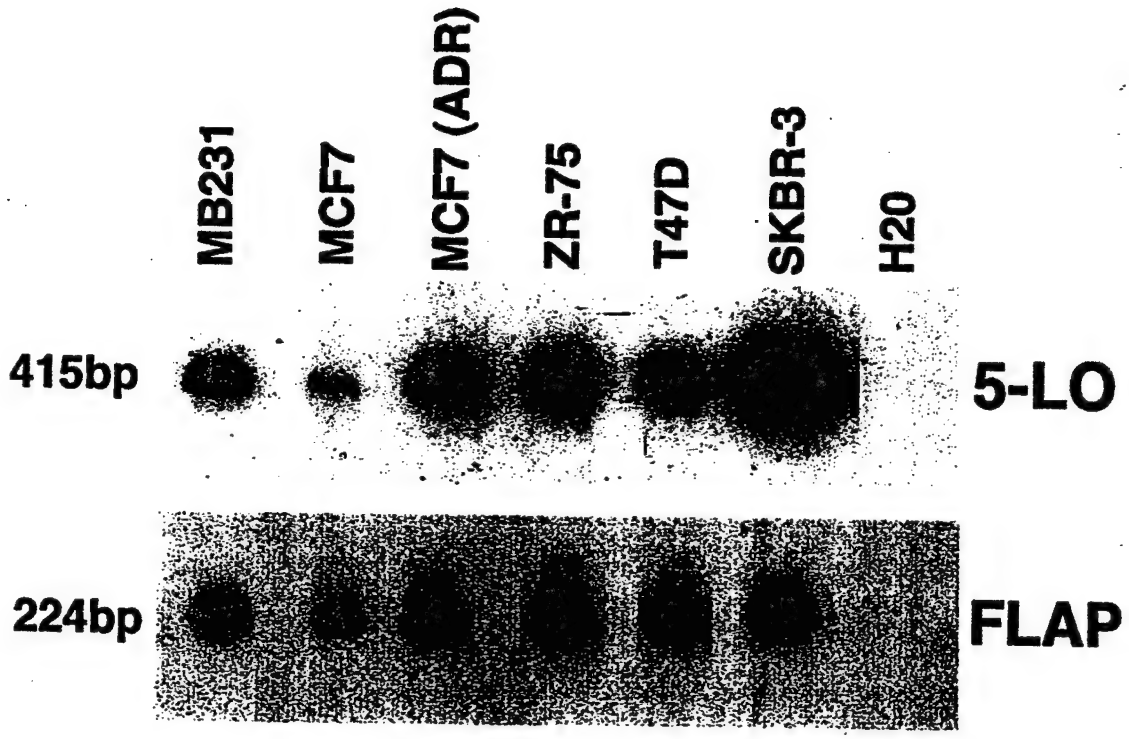


Figure 3a

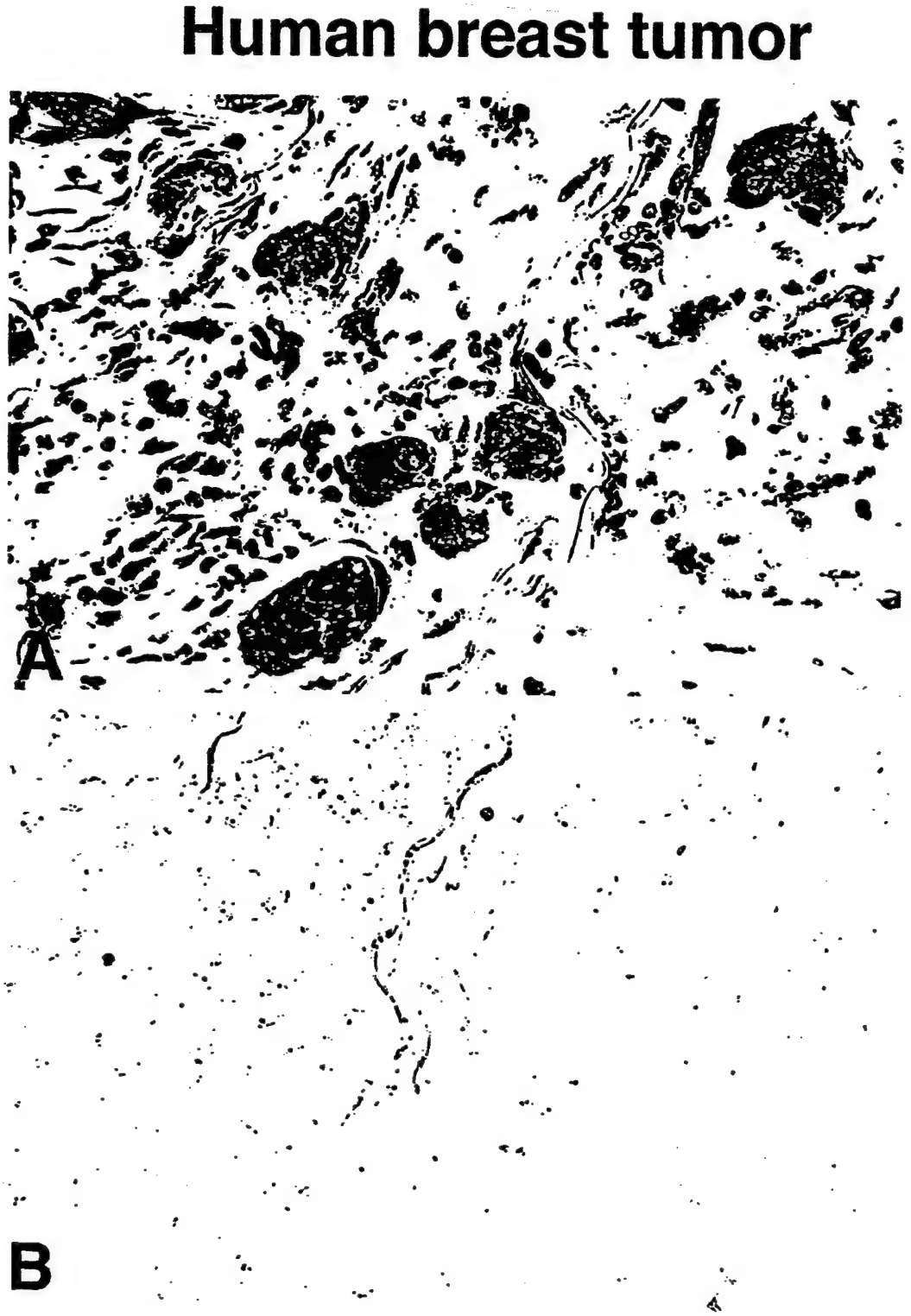
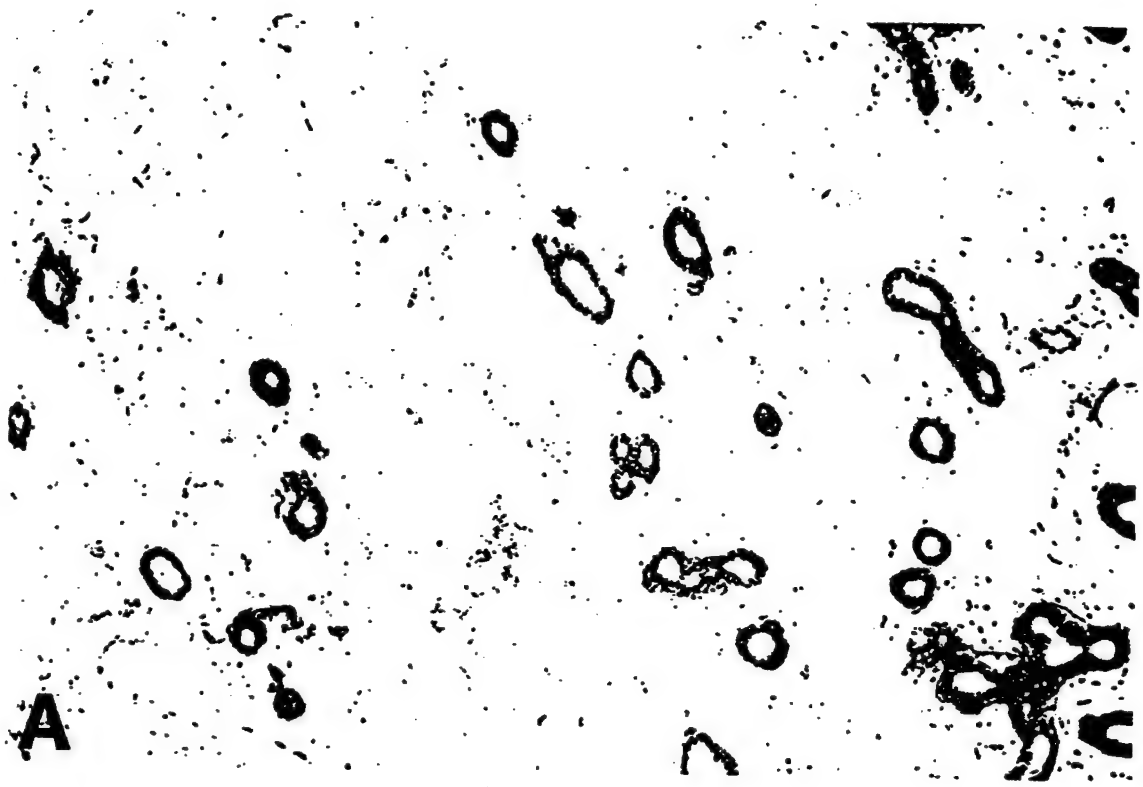


Figure 3b

Normal human breast tissue



B

Figure 4

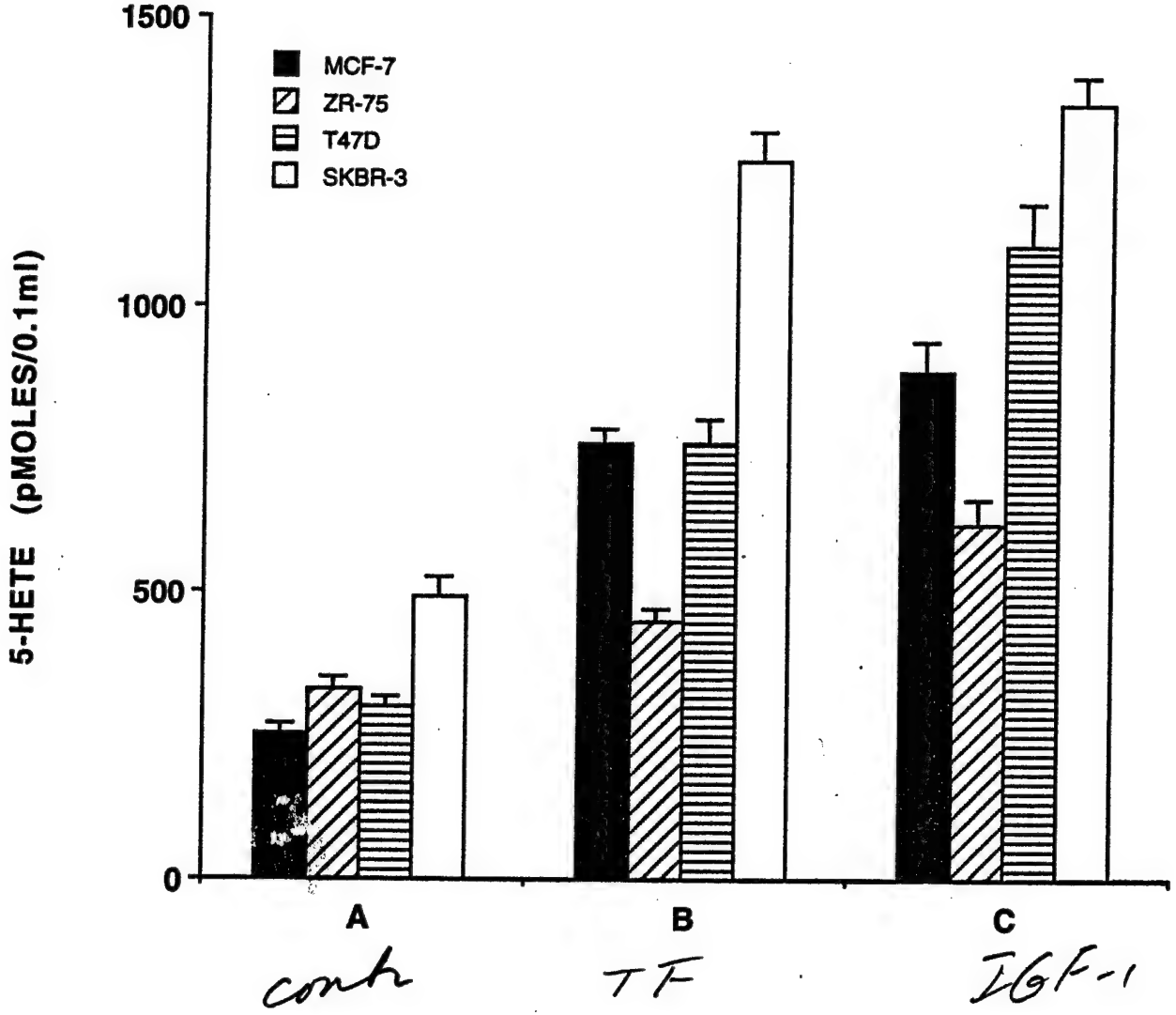

1500
1000
500
0
5-HETE (pMOLES/0.1ml)
MCF-7
ZR-75
T47D
SKBR-3
A
B
C
cont
TF
IGF-1

Figure 5

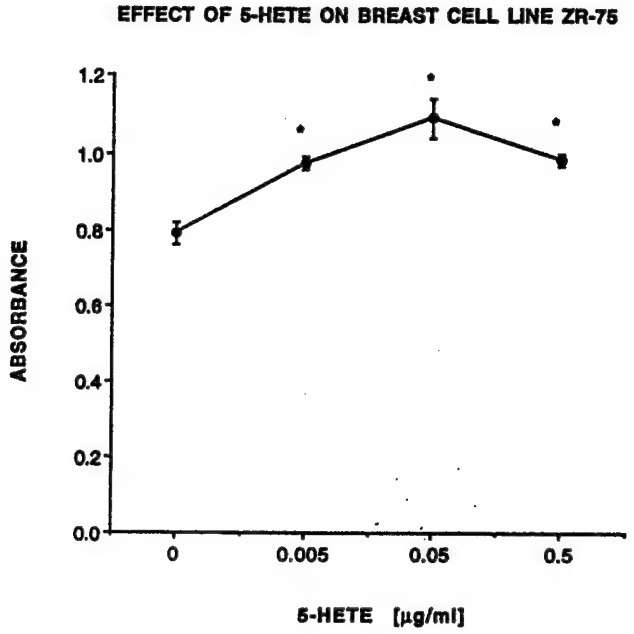

EFFECT OF 5-HETE ON BREAST CELL LINE ZR-75
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.005
0.05
0.5
5-HETE [µg/ml]

Figure 6

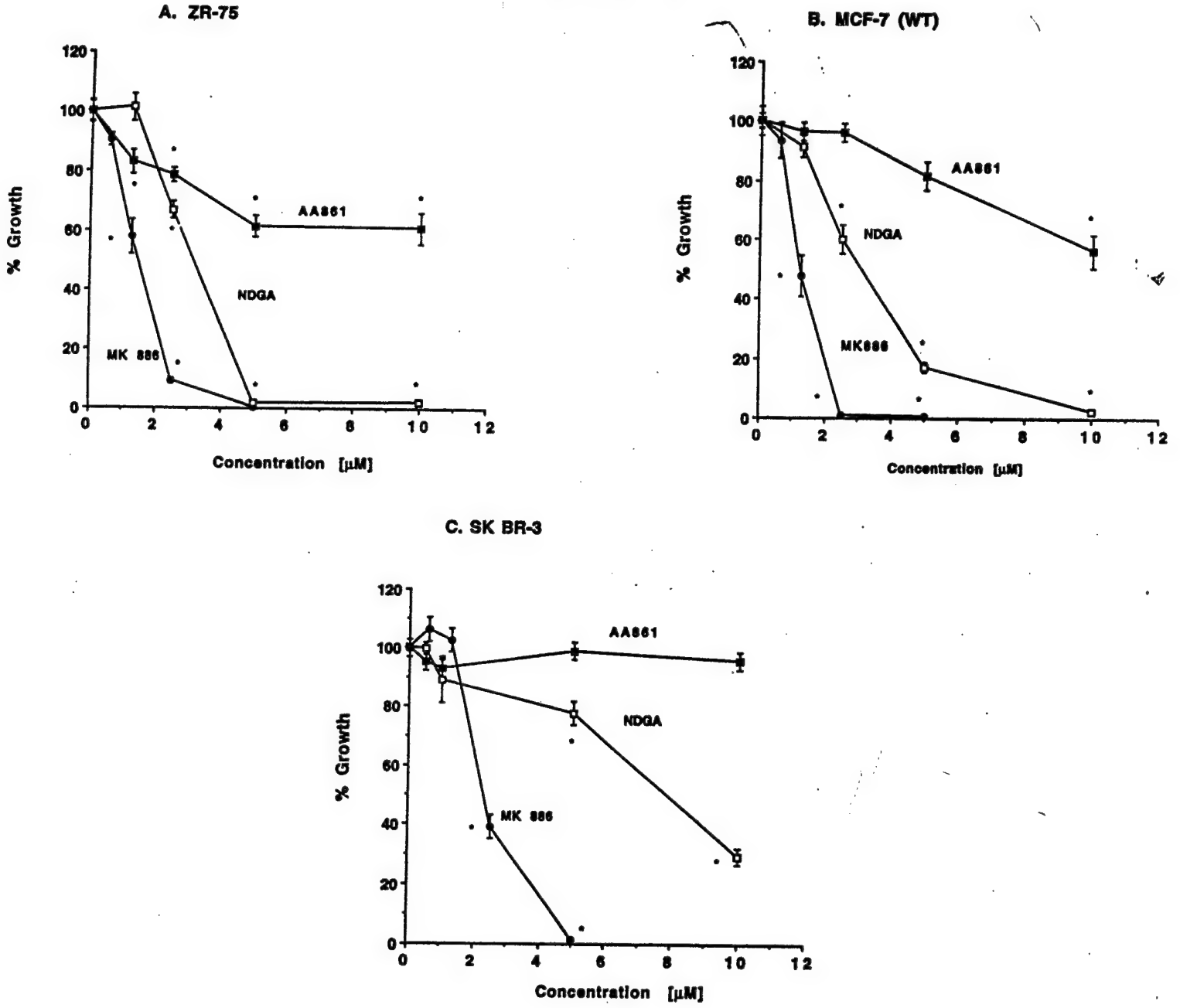

A. ZR-75
% Growth
Concentration [µM]
AA861
NDGA
MK 886
B. MCF-7 (WT)
% Growth
Concentration [µM]
AA861
NDGA
MK886
C. SK BR-3
% Growth
Concentration [µM]
AA861
NDGA
MK 886

Figure 7

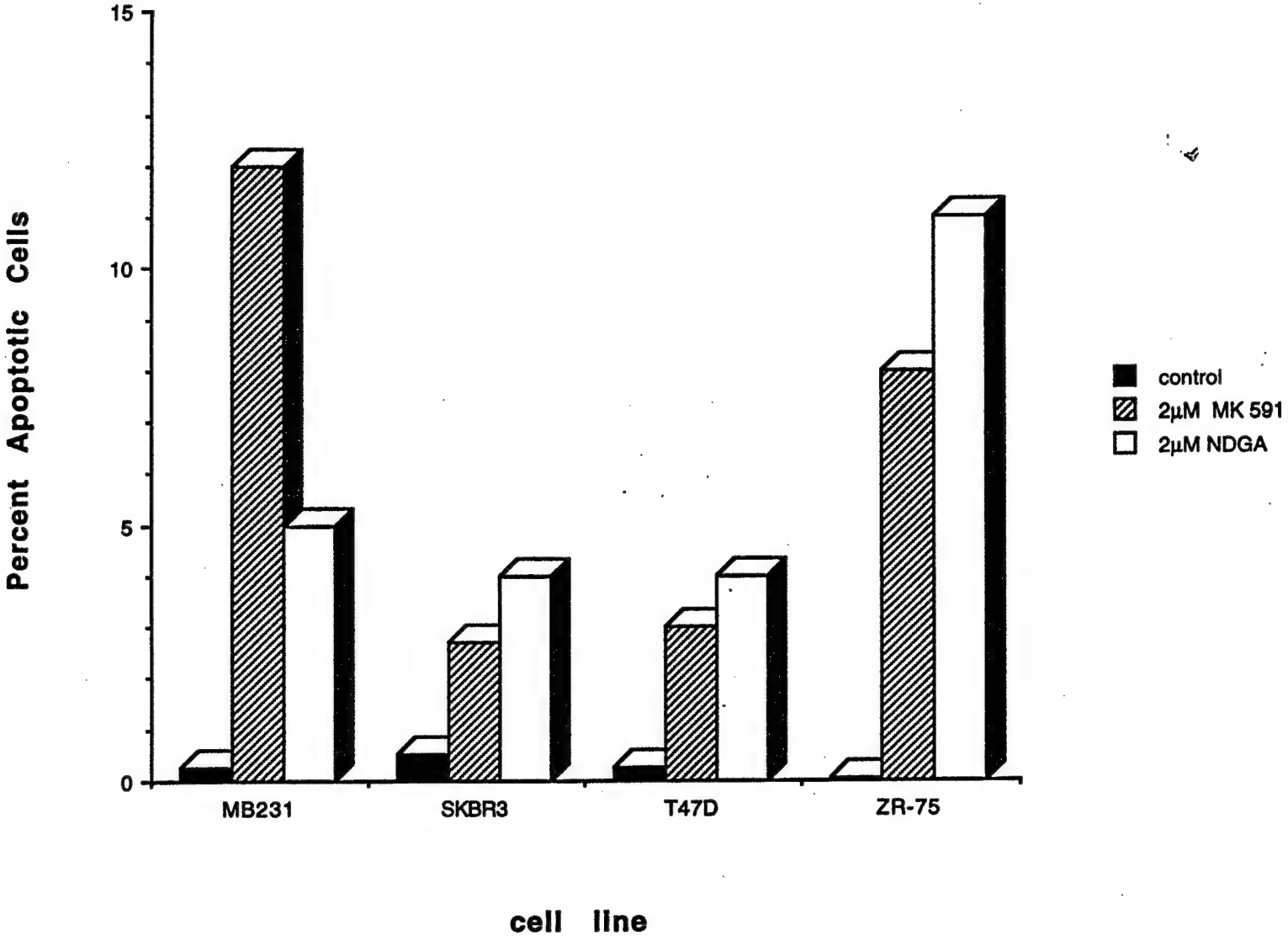

15
10
5
0
Percent Apoptotic Cells
MB231
SKBR3
T47D
ZR-75
cell line
control
2μM MK 591
2μM NDGA

Figure 7(Cont)

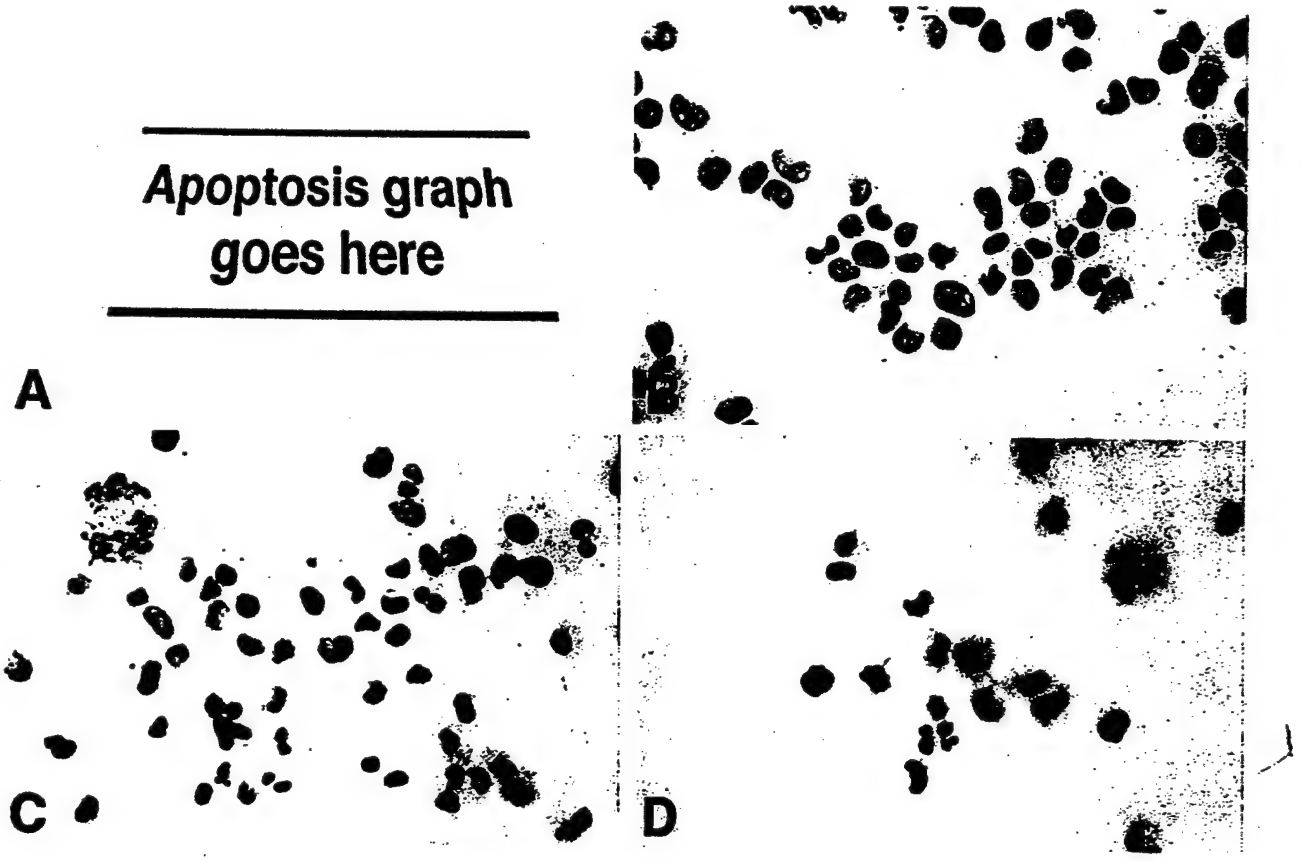
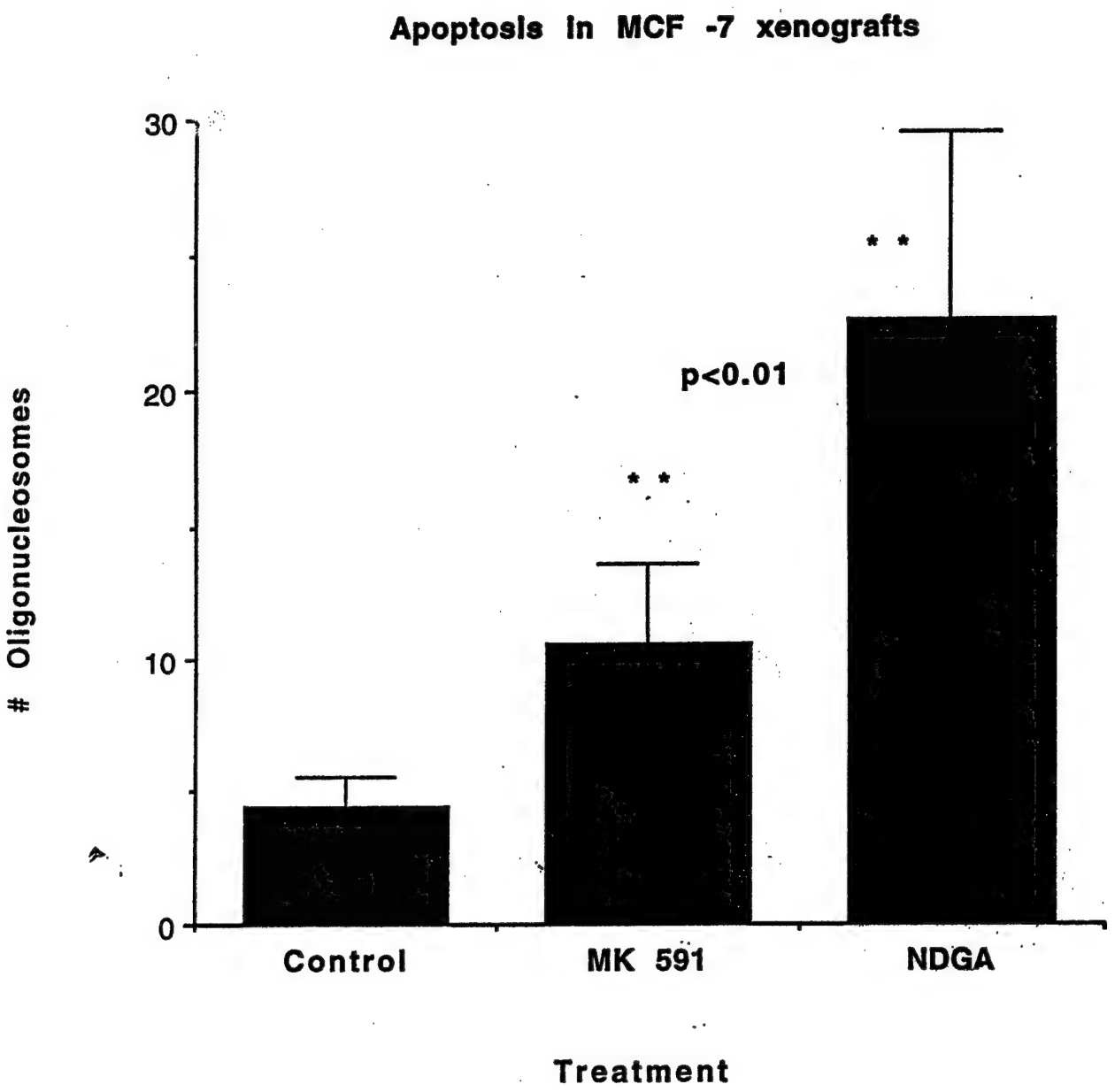


Figure 8


Apoptosis in MCF -7 xenografts
30
20
10
0
Oligonucleosomes
p<0.01
* *
* *
Control
MK 591
NDGA
Treatment

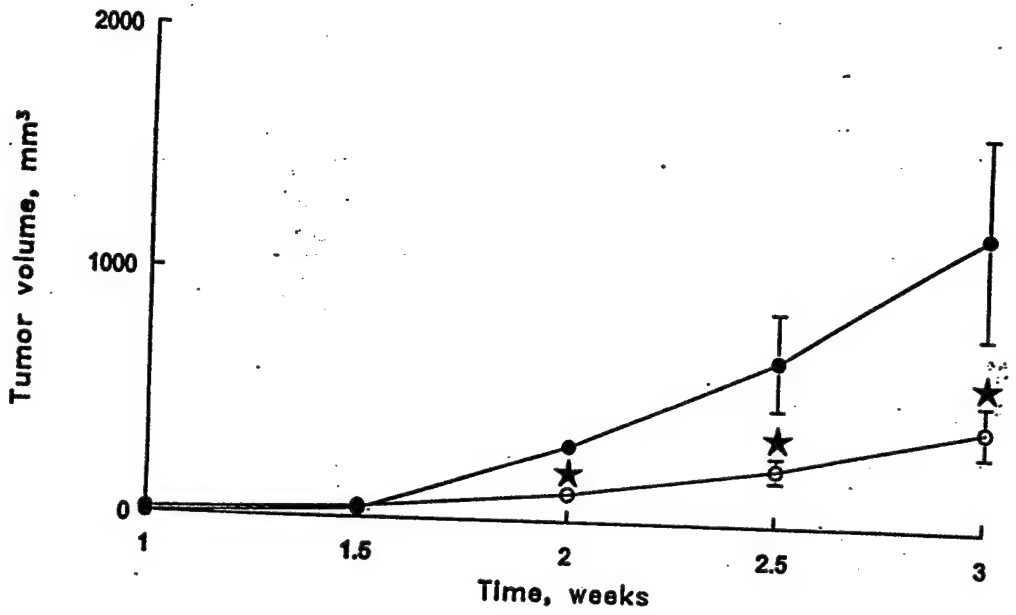
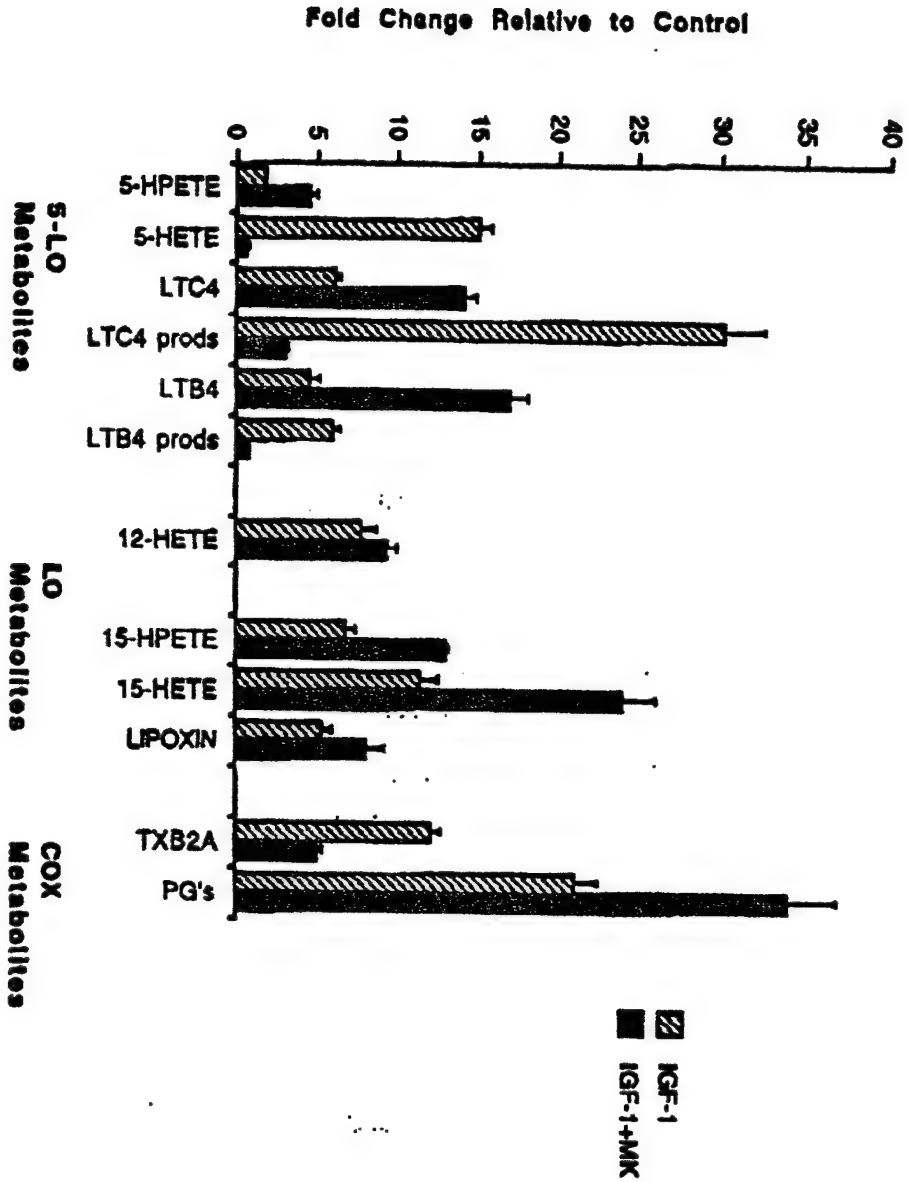


Table 4

Figure 9


Fold Change Relative to Control
0
5
10
15
20
25
30
35
40
5-HPETE
5-HETE
LTC4
LTC4 prods
LTB4
LTB4 prods
12-HETE
15-HPETE
15-HETE
LIPOXIN
TXB2A
PG's
5-LO Metabolites
LO Metabolites
COX Metabolites
IGF-1
IGF-1+MK

Control of human breast cancer cell growth by manipulation of arachidonate metabolism.

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Key Words: 5-LO, Arachidonic Acid pathway, bioactive lipids, MCF-7, bone marrow cells, signaling

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ABSTRACT

Arachidonate metabolites are important regulators of cell growth in human breast cancer. Production of bioactive lipids is initiated by the enzyme phospholipase A2 which releases arachidonic acid (AA); AA is rapidly metabolized by cyclooxygenases (CO) or lipoxygenases (LO) to other highly potent lipids. In the present study we used inhibitors which blocked specific pathways in arachidonate metabolism. Many of these drugs proved to be equally effective in blocking proliferation in both MCF-7 wild type (WT) and MCF-7 multidrug resistant (ADR) human breast cancer cell lines and were non toxic to cultures of human bone marrow cells. Inhibitors of 5-LO pathway (MK-591, MK-886, and AA-861) were most effective in blocking proliferation of both of these breast cancer cell lines while CO inhibitors (indomethacin, aspirin, etc.) were not effective. Curcumin, an inhibitor of both cyclooxygenase and lipoxygenase pathways of eicosanoid metabolism, was equally effective in blocking proliferation of the multidrug-resistant cells, MCF-7 ADR^{10} and WT cells. Cell cycle studies showed curcumin to arrest cells at G1 leading to apoptosis. Protein kinase C (PKC), the activation of which is intertwined with arachidonate metabolism, also proved to be a good target since inhibitors of PKC effectively blocked proliferation in these cultures. PCA-4248, an inhibitor of platelet activating factor generation (a byproduct of arachidonate release), was an effective antiproliferative agent. Thapsigargin, a drug which induces the release of stored calcium, blocked proliferation in MCF-7 WT (IC50= 5 nM) but was ineffective with MCF-7 ADR^{10} cells. Inhibitors that effectively block the proliferation of breast cancer cells showed varying degrees of toxicity to cultures of human bone marrow cells. We observed greater toxicity to bone marrow cells using inhibitors that interfered with the utilization of arachidonic acid in contrast to those that blocked utilization of its downstream metabolites. MK-591, MK-886, PCA-4248, Curcumin and AA-861 blocked proliferation of breast cancer cells but showed little toxicity to bone marrow cells and may be potentially useful in human breast cancer therapy.

INTRODUCTION

Epidemiologic investigations have suggested an association of dietary fat intake with breast cancer risk. Bioactive lipids generated from these fat metabolites are known to increase proliferation in cancer cells. Various studies have suggested dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids, particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Phospholipase A2 (PLA2) is the family of enzymes which specifically hydrolyzes the 2-acyl position of glycerophospholipid. It has been reported that the concentration of PLA2 was elevated in the lungs, breasts, and the digestive organs of patients with malignant tumors and that the incidence and magnitude of the elevation increased with advanced cancer stage [4,5]. In our previous work with wild type (WT) and drug-resistant (MCF-7 ADR^{10}) MCF-7 cells, we observed PLA2 activity with specificity toward either linoleoyl or arachidonyl phosphatidylinositol [6]. PLA2's are usually most efficient with polyunsaturated fatty acids in the SN-2 position, which result in the release of arachidonic acid (AA). Activation of AA metabolism is initiated by the release of AA from the phospholipid pool by the enzyme phospholipase A2 (Axelrod et al. 1988). AA is metabolized through the cyclooxygenase pathway which results in prostaglandins production (Boyle et al. 1994) or through the 5-lipoxygenase (5-LO) pathway, which results in the production of leukotriene (Henderson, 1994). Both prostaglandins and leukotriene directly stimulate the growth of malignant cells (Lee and Ip, 1992; Snyder et al. 1989).

Metabolism of exogenous arachidonic acid by lipoxygenase or cyclooxygenase pathways produces a myriad of highly potent bioactive lipids which include leukotrienes, HPETEs, HETEs, and prostaglandins. Many of these metabolites have been shown to play a significant role in cancer cell growth. The arachidonate-derived eicosanoids PGE2, LTB4, and 5-, 12-, and 15-HETEs have been shown to be significantly higher in human

breast cancer cells than control cells [refs]. In Swiss 3T3 cells, stimulation of DNA synthesis occurs predominantly by activation of arachidonic acid release, followed by its oxidation to PGE2 and stimulation of adenylyl cyclase [refs]. Metabolites of arachidonic acid and linoleic acid served as regulators of the EGF transduction system in Syrian hamster embryo fibroblasts [refs]. Initiation of growth of human myeloblastic leukemia cells is dependent upon the increased formation of AA and its derivatives, formed primarily via the lipoxygenase pathway and the initiation of growth in these cells was followed by the rapid release of AA, HETEs and phospholipids into the culture medium [refs]. The effects of lipoxygenase and cyclooxygenase metabolism inhibitors on a human gastric cell line derived from a stomach tumor was suppression of cell proliferation [refs]. The consequent alteration in PKC, catalyzed by phospholipase(s) activity in endothelial cells, regulates the growth-dependent changes in AA release [refs].

Avis et al. reported that exogenous addition of 5-HETE was found to stimulate lung cancer growth in vitro [Avis, 1996 #16]. When selective antagonists were used to inhibit 5-lipoxygenase metabolism, significant growth reduction resulted in a number of lung cancer cell lines. Similarly, LTB4 and 12(R)-HETE significantly increased proliferation of two colon carcinoma cell lines, HT-29 and HCT-15 [Bortuzzo, 1996 #17]. However, isomers of these two compounds such as LTB5 and 12(S)-HETE failed to affect the proliferation rate of these two cell lines. This demonstrates the importance of specificity in cancer cell proliferation. Epidemiology studies show that death rates from colon cancer decreased 40% for individuals who took aspirin (AA inhibitor) more than 16 times/month [7]. The use of inhibitors to manipulate AA pathways will help us better understand the function of elevated PLA2 levels in cancer cells, which may lead to the discovery of new anti-cancer drugs.

In the present study we have examined the effect of various inhibitors of key signaling pathways on growth of breast cancer cells. We show that inhibitors of the 5-LO pathway can block growth of breast cancer cells , especially the drug resistant MCF-7 ADR line very effectively. Inhibitors of the PKC pathway were also able to block the growth of these cells. The toxicity of these inhibitors were then tested on the growth of bone marrow cells. The selection of some of these inhibitors might be an alternative

treatment regimen for the breast cancer patients especially those that develop drug resistance during their course of treament.

MATERIALS AND METHODS

Materials. MK-591 was a gift from Merck-Frost, Pointe Claire, Quebec, Canada. All other inhibitors were purchased from BIOMOL Research Laboratories, Inc.

Cells. The human breast cancer MCF-7 Wild Type (WT) and its Adriamycin-Resistant, (MCF-7 ADR[10]) cells were obtained from Kenneth Cowan (NCI, NIH Bethesda, MD). The cells were grown in Improved Modified Essential Medium (IMEM)[4] containing 8% fetal bovine serum, 50,000 units/liter penicillin and 5,000 μg/l streptomycin at 37°C with 5% CO_2.

Human bone marrow mononuclear cells were obtained from healthy volunteer donors arranged by Poietic Technologies Inc. and used for colony assays without any prior treatment. The stroma colonies were grown in IMDM-based Long Term Culture Media[5] (LTCM) containing 25% horse serum (Hyclone) with 5% CO_2 and 100% humidity at 37°C.

Methyl (H^3)-thymidine incorporation. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR[10] cultures was performed in 96-well plates. Cells were plated 15,000 cells/well in 0.2 mL IMEM culture fluid[1] and incubated overnight. Inhibitors were added (50 μl) to achieve the indicated concentration and incubated for three days. During the last 18 hours, Methyl(H^3)-thymidine (1μCi / well) was added. The cells were trypsinized and harvested using Packard Unifilter System. The filter plates were dried in the air. Then, 40μL of Packard Microscint 0 scintillation cocktail was added to each well and the filter plates were counted using Packard Top-count.

Colony Assay. Human bone marrow stroma colony studies were performed using human bone marrow mononuclear cells[2]. Cells ($2x10^5$ cells/well) were plated in 4-well plates with 5 ml LTCM and inhibitors were added on the second day. The media was changed with fresh media either with (continuous treatment) or without drugs (pulse) every week. The stroma colonies were stained on the second and fourth week of

treatment with HEMA 3 (differential hematology stain, CMS). Colonies with diameters larger than 1 mm were counted and the size of the each colony was measured.

The hematopoietic progenitor colony assay was performed using Methylcellulose-based Colony Cocktail from Stem Cell Technologies, Inc. (HCC-4434) containing 30% Fetal Bovine Serum; 50 ng/ml rh Stem Cell Factor; 10 ng/ml rh GM-CSF; 10 ng/ml rh IL-3; and 3 units/ml rh Erythropoietin. Inhibitors were premixed with the cocktail (4.5 ml) and were added with the cells and plated in a 35mm diameter gridded tissue culture plate (Nunc. Inc.). After a two week incubation period, the hematopoietic colonies were counted using an inverted phase microscope with 40X magnification. Using the standard criteria developed by Stem Cell Technologies Inc. (Atlas of Human Hematopoietic Colonies), the colonies were classified into categories that included: CFU-GM, BFU-E , CFU-E, CFU-Mix, and CFU-GEMM.

Both stroma colony and hematopoietic colony assay experiments were performed with four replicates and repeated at least three times with different lots of cells. Regardless of the lot of the cells, the same trends were observed.

RESULTS

Effect of Lipoxygenase/Cyclooxygenase inhibitors on breast cancer cell growth: Cells were plated in 96 well plates in the presence of various inhibitors of the arachidonic acid pathway and their growth was measured by 3H Thymidine incorporation. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR[10] cultures with lipoxygenase inhibitors is shown in figure 1. Inhibitors of lipoxygenase (LO) pathways were most effective at blocking proliferation. The effect of MK591, MK886 and NDGA was compared between the MCF-WT (Fig 1 A) and MCF-7 ADR cells (Fig 1 B). There was a concentration dependent decrease in growth of these cells in the presence of these inhibitors. Curcumin, AA861, and ketoconazole also blocked cell growth of these breast cancer cells (Fig 1 C, D). Curcumin, a dual inhibitor of 5-lipoxygenase and cyclooxygenase, was much more effective (ca. 12 fold) at blocking proliferation of the multidrug-resistant cells, MCF-7 ADR[10] compared to MCF-7 wild type (WT) cells.

Curcumin, an agent with anti-inflammatory and anti-oxidant properties [Xu, 1997 #8]. showed maximum inhibition of growth of breast cancer cells at very low concentrations.

However, cyclooxygenase (CO) inhibitors were not effective in blocking the growth of these breast cancer cells (Table 1). Since metabolites of 5-LO has properties of a co-growth factor, it is possible that decreases in 5-LO products inhibit breast cancer cell proliferation. It is also possible that decreases in the ratio of lipoxygenase to cyclooxygenase products are responsible for the inhibition.

Effect of PKC Inhibitors on growth of breast cancer cells:

Protein Kinase C (PKC) has become an increasingly important signaling kinase in cancer research because it serves as a receptor for phorbol esters, potent tumor promoters [Hannun, 1989 #11]. We found that some PKC inhibitors such as sphingosine and chelerythrine chloride were very effective at blocking proliferation of MCF-7 WT and MCF-7 ADR^{10} cells. Inhibition of proliferation in WT or MCF-7 ADR^{10} cultures with PKC inhibitors is shown in figure 2. Sphingosine disrupts sphingolipid metabolism by preventing PKC from interacting with DAG and phorbol esters which may lead to the accumulation of lysosphingolipids and the consequent inhibition of PKC [8a]. Chelerythrine chloride causes cell cycle arrest in G2 by selectively inhibiting and degrading betaII protein kinase C, which must be activated for mitotic nuclear lamina diassembly and entry into mitosis [Thompson, 1996 #10].

Other PKC inhibitors such as H7 were ineffective in this system. H7 is a non-selective inhibitor of protein kinases which inhibits PKA, PKG, MLCK, and PKC activity [Nixon, 1991 #13].

Regulation of breast cancer cell growth by PAF inhibitors:

PCA-4248 inhibits PAF (a byproduct released from arachidonate metabolism) binding to human platelet and PMNL receptors. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures with PCA-4248 and thapsigargin is shown in Figure 3. Blocking PAF binding results in the accumulation of arachidonate metabolism products, which may account for the inhibition of proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures by PCA-4248. Thapsigargin induces the release of intracellular Ca^{2+} without hydrolysis of

inositolphospholipids and down-regulates the EGF receptor. It inhibits drug resistant MCF-7 ADR[10] cells effectively at very low concentrations. However for MCF-7 wild type cells, there was a very small degree of inhibition at the same concentration.

Human Bone Marrow progenitor colonies in the presence of inhibitors:
For any drug to be useful for chemotherapy it is important to study the toxicity of the drugs on bone marrow cells. To determine the toxicity of these inhibitors, the ability of human bone marrow cells to form colonies was studied. Human bone marrow stroma colonies with one week of treatment with the inhibitors are shown in Figure 4 (two week colonies) and Figure 5 (four week colonies). The total number of stromal colonies, the average area of each colony, and the percentage coverage of the plate did not change in the presence of MK-591, MK-886, and PCA-4248 at IC_{50}, when compared to the control values. However, the stroma colonies were wiped out even with concentrations of the inhibitors lower than IC_{50} for NDGA and sphingosine. Lipoxygenase inhibitors (MK-591 and MK-886) did not have any toxic effect on the formation of stroma colonies. PKC inhibitors stopped the colony growth at a concentration lower than IC_{50}. However, PAF Binding Inhibitor PCA-4248, did not affect the growth of stroma colonies when the concentration was at IC_{90}. When the human bone marrow cells were treated for two weeks or four weeks continuously with the inhibitors, the growth of the colonies show similar trends to those treated for one week (Figure 6 and 7).

Figure 8 shows the total number of hematopoietic colonies in the presence of these inhibitors. The distribution of these colonies categorized as BFU-E, CFU-E, CFU-GM, CFU-mix and CFU-GEMM is summarised in Table 2. Again MK-591, MK-886, and PCA-4248 showed no toxicity on the hematopoietic colonies. The relative population of the hematopoietic colonies in these treated samples were similar to the control. NDGA and Chelerythrine chloride were toxic to hematopoietic colonies only at higher concentrations. Sphingosine and curcumin were very toxic to hematopoietic colonies even at lower concentrations. Thus the MK drugs and PCA are some of the drugs that may have some use in the clinics in future becuase they are safe and effective in blocking the growth of breast cancer cells.

DISCUSSION

There is a constant need for additional therapeutic strategies to combat the disease in order to improve the quality of life and prolonge survival for women with breast cancer. Bioactive lipids play an important role in the growth and development of the normal mammary gland . Understanding the regulatory role of these lipids on the control of the epithelial cell population is extremely important. Dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Signal transduction explains the molecular events by which extracellular signals elicits an intracellular response. The generation of a variety of lipid signal transduction molecules from hydrolysis of membrane phospholipids is an early response. Several types of signal transduction pathway inhibitors effectively blocked proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures. Lipoxygenase (LO) inhibitors NDGA, MK591, AA-861 and MK886 were equally effective at blocking proliferation for MCF-7 WT and MCF-7 ADR^{10} cells.

Curcumin has been shown to strongly inhibit the LO pathway and weakly inhibite the CO pathway. Curcumin inhibits proliferation by blocking the action of the Thymidine Kinase enzyme which is necessary for cell cycle progression through the S-phase [Singh, 1996 #7]. In immortalized NIH 3T3 and malignant cancer cell lines, curcumin induced cell shrinkage, chromatin condensation, DNA fragmentation, and characteristics of apoptosis. In the present study it inhibits the proliferation for both MCF-7 WT and MCF-7 ADR^{10} cells. However, the IC_{50} for MCF-7 ADR^{10} cells (12μM) is much smaller than that for MCF-7 WT cells (90μM). Interestingly, curcumin effectively blocked proliferation in only MCF-7 ADR^{10} much more than in MCF-7 WT cells. However Ketoconazole was more effective with MCF-7 WT cells than MCF-7 ADR^{10} cells

The cyclooxygenase (COX) enzyme is an important enzyme because it catalyzes the initial reaction of arachidonate metabolism that leads to the formation of prostaglandins, thromboxane, and prostacyclin [Gierse, 1996 #14]. Recently, a second form of the cyclooxygenase (COX) enzyme, COX-2, has been isolated. A single amino acid difference in the active site (valine 509 to isoleucine) and a series of differences in the active site confers selectivity for COX-2. COX-2, which can be induced by cytokines and growth factors, is linked to inflammatory cell types and tissues whereas COX-1's course of action resides primarily in the stomach and kidney [Gierse, 1996 #14]. It is possible that a selective COX-2 inhibitor may eliminate the side effects associated with COX-1 while still mainitaining COX-2 inhibition and vice versa. In the present study we did not observe any significant inhibition of breast cancer cell growth in the presence of COX inhibitors.

Since PKC is such an important regulator of cell growth and differentiation, the finding that some PKC inhibitors possess antiproliferative properties can be a very exciting area of cancer research [Budworth, 1994 #18]. PKC, which occurs as a family of isoenzymes that share similar structural features, is regulated by calcium, phospholipids, and sn-1,2-diacylglycerol (DAG). Some PKC inhibitors such as Sphingosine and Chelerythrine chloride were effective at inhibiting growth of both breast cancer cell lines while others such as H7 were not. We found that H7, which many people believe to be a potent inhibitor, to be ineffective against proliferation. As we know, there are many isozymes of PKC so it is possible that only certain isomers play an important role in MCF-7 WT cell proliferation. The problem with H7 is that many of them only possess modest degrees of specificity for PKC [Budworth, 1994 #18]. Thus there is a causal relationship between inhibition of PKC and cancer cell growth arrest.

An inhibitor of PAF (PCA-4248) effectively blocked proliferation of MCF-7 WT and MCF-7 ADR[10]. Thapsigargin, a down- regulator of the EGF receptor and PI3 kinase was very effective only with MCF-7 WT cells. Inhibitors of the LO pathway or specifically the 5-LO pathway were most effective at blocking proliferation while cyclooxygenase (CO) inhibitors were not effective. Blocking both LO and CO pathways in WT cells using ETYA, phenidone or curcumin did not inhibit the proliferation. In

general, these inhibitors were more effective blockers of CO than LO pathways. We have previously observed {Avis et al, 1996} that AA metabolism in the presence of aspirin was shifted to large increases in LO products. This might have occurred in the presence of these dual inhibitors.

NDGA, MK-591, and MK-886 block arachidonic acid metabolism pathway at different stages and all effectively inhibit the proliferation of MCF-7 WT and MCF-7 ADR[10] cultures. MK-591 and MK-886 did not exhibit any toxicity on either human bone marrow stroma colonies or on human hematopoietic colonies at a concentration lower than IC_{90} . However, NDGA completely blocked stroma colony formation even though the hematopoietic colonies still survived at a concentration lower than IC_{70}. This could be due to the fact that it blocks the initial conversion from AA to any/all of the lipoxygenases and their products. PCA-4248, an inhibitor of PAF, did not have an effect on either stroma colonies or hematopoietic colonies at a concentration below IC_{90}. Sphingosine and Curcumin completely stopped the growth of stroma colonies and hematopoietic colonies even when the concentration was at IC_{50} for WT cells suggesting that these are highly toxic drugs (What conc was used?). Chelerythrine chloride was mildly toxic, which changed with increasing concentration. Our results indicate that MK-591, MK-886, and PCA-4248 could be good candidates for medical clinical trails.

Use of these inhibitors of different signaling pathways gives us a better understanding of the mechanism of action of the bioactive lipids in breast cancer. By blocking one of these pathways we were able to block the growth of breast cancer cells, especially the cells that develop drug resistance. However not all of these inhibitors serve as a potential therapeutic agents because of their toxicity to the bone marrow cells. The MK drugs which block 5-LO pathway and block cell growth, were not toxic to the bone marrow cells and therefore may be of some use in controlling the growth of breast cancer cells openning new avenues for therapeutic intervention.

Figure 1. Inhibition of proliferation in (A and C) WT or (B and D) ADR MCF-7 cultures with lipoxygenase inhibitors; (●) MK591, (■) MK886, (▲) NDGA for A and B; (▼) curcumin, (◆) ketoconazole, and (➢) AA881 for C and D. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 2. Inhibition of proliferation with PKC inhibitors in (A) WT and (B) ADR MCF-7 cultures; (●) sphingosine, (■) chelerythrine chloride. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 3. Inhibition of proliferation in (●) WT and (◆) ADR MCF-7 cultures with (A) PAF binding inhibitor PCA-4248 and (B) tumor promoter thapsigargin. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 4. Human bone marrow stroma colonies with one week ~~treatment after~~ a 2 weeks incubation. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 5. Human bone marrow stroma colonies with one week ~~treatment after a~~ 4 weeks ~~incubation~~. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 6. Human bone marrow stroma colonies with continuous treatment ~~after a~~ for 2 weeks ~~incubation~~. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 7. Human bone marrow stroma colonies with continuous treatment ~~after a~~ for 4 weeks ~~incubation~~. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 8. The treatment of human bone marrow cells with drugs at different concentrations.

Table 1. The inhibitors of signal transduction pathways that are not effective for MCF-7 cells under the present conditions.

Name	Function of Inhibition	Concentration range
ETYA	Arachidonic Acid uptake, Arachidonic acid specific and nonspecific acyl-CoA synthetase, PLA2, cytochrome P-450, LO/CO	20-50μM
Phenidone	LO/CO	100-400μM
Aspirin	CO	10-30μM
Benzylimidazole	Induces cytochrome P-450 isozymes including lauric acid, Thromboxane A2 synthase	5-50μM
H7	PKA, PKG, MLCK, and PKC	10-30μM
Tyrphostin 1	Inactive tyrphotin	10-40μM
Tyrphostin 23	EGF receptor kinase activity	10-40μM
Gossypol	RBL-1,5-LO and 12-LO, PAF, and leukeotriene-induced guinea pag parenchyma contractions	20-50μM
NMDA	Excitatory amino acid	30-50μM

LO: Lipoxygenase; CO: Cyclooxygenase

Table 2. Hematopoietic colony distribution

Inhibitors	BFU-E	CFU-E	CFU-GM	CFU-MIX	CFU-GEMM
control	30	43	55	13	4
MK591-16	24	24	70	14	3
MK591-40	37	26	63	12	3
MK591-70	10	16	24	0	0
NDGA-16	26	25	61	12	4
NDGA-40	4	4	18	1	0
NDGA-70	0	0	0	0	0
PCA-13	29	24	93	12	3
PCA-38	29	15	71	4	2
CHEL-10	26	28	83	21	4
CHEL-20	24	30	40	2	2
CHEL-40	11	18	0	0	0
MK-886-8	33	25	91	12	4
MK886-30	28	21	56	8	2

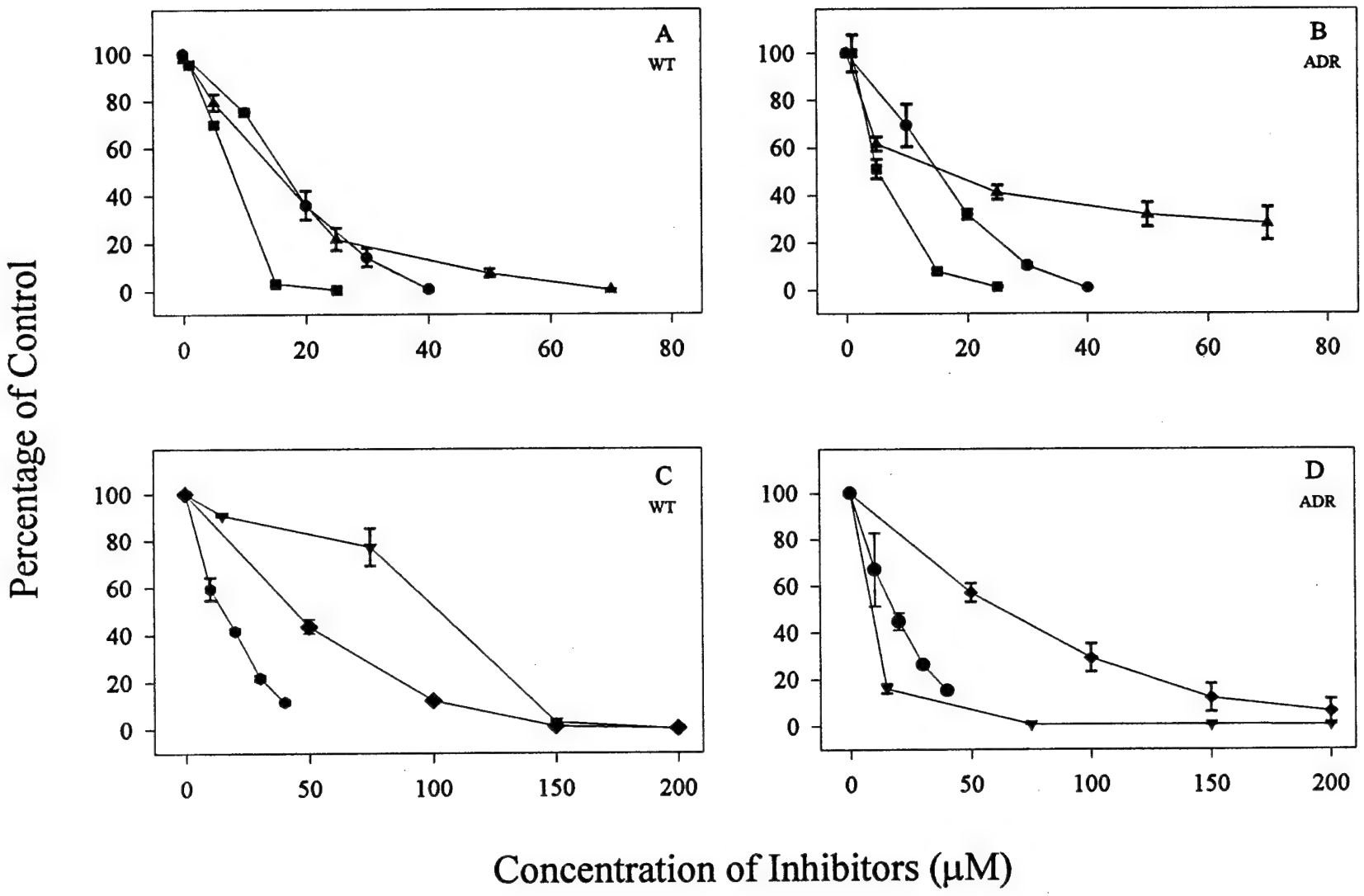
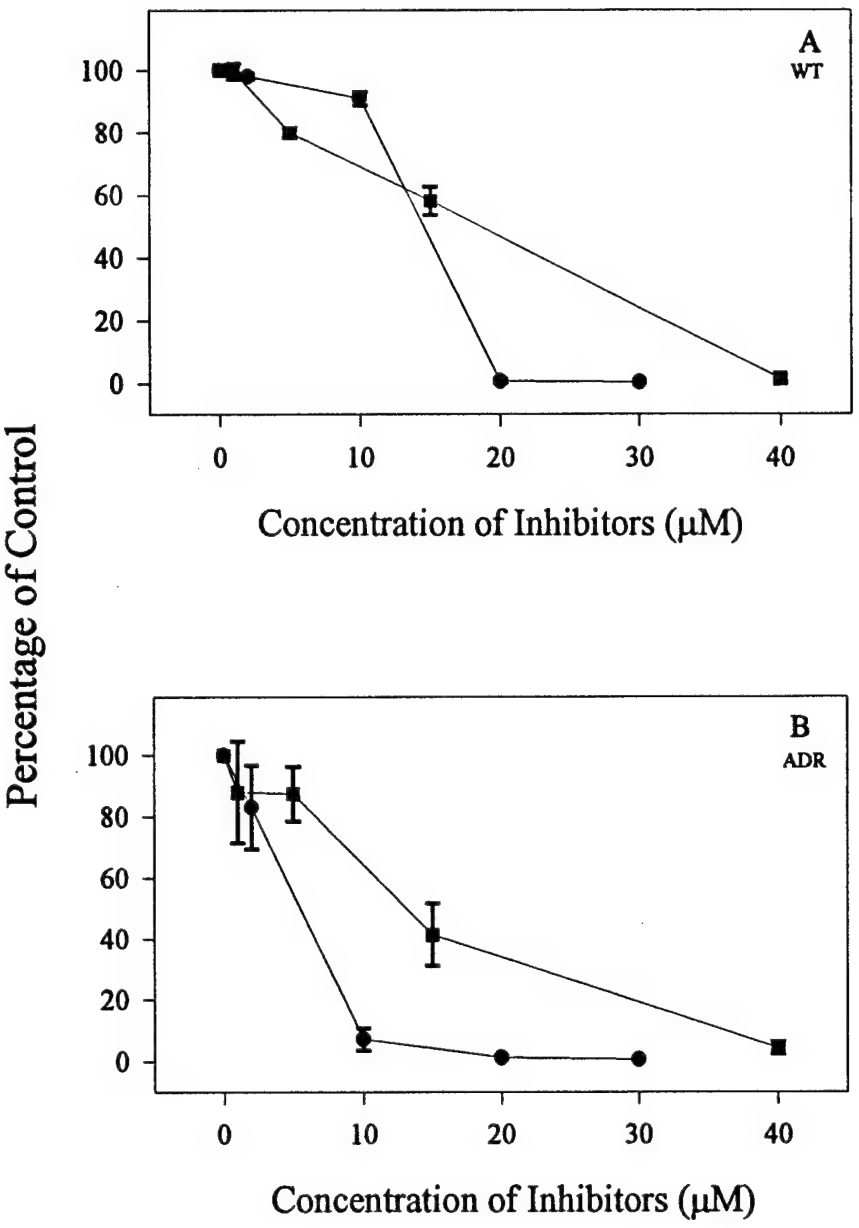

A
WT
B
ADR
C
WT
D
ADR
Percentage of Control
Concentration of Inhibitors (μM)

Figure 1.


A
WT
100
80
60
40
20
0
0
10
20
30
40
Concentration of Inhibitors (μM)
Percentage of Control
B
ADR
100
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20
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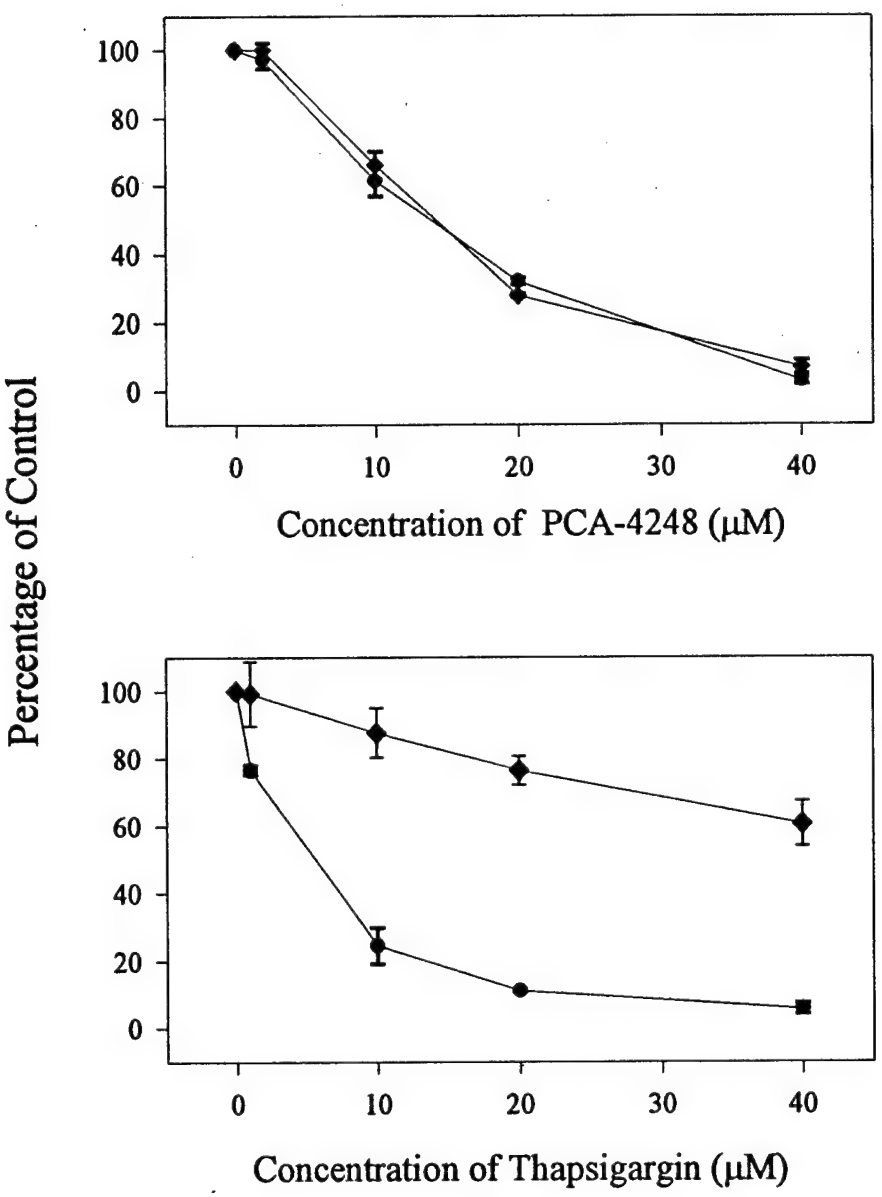
10
20
30
40
Concentration of Inhibitors (μM)

Figure 2.


Percentage of Control
100
80
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0
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10
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30
40
Concentration of PCA-4248 (μM)
100
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60
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20
0
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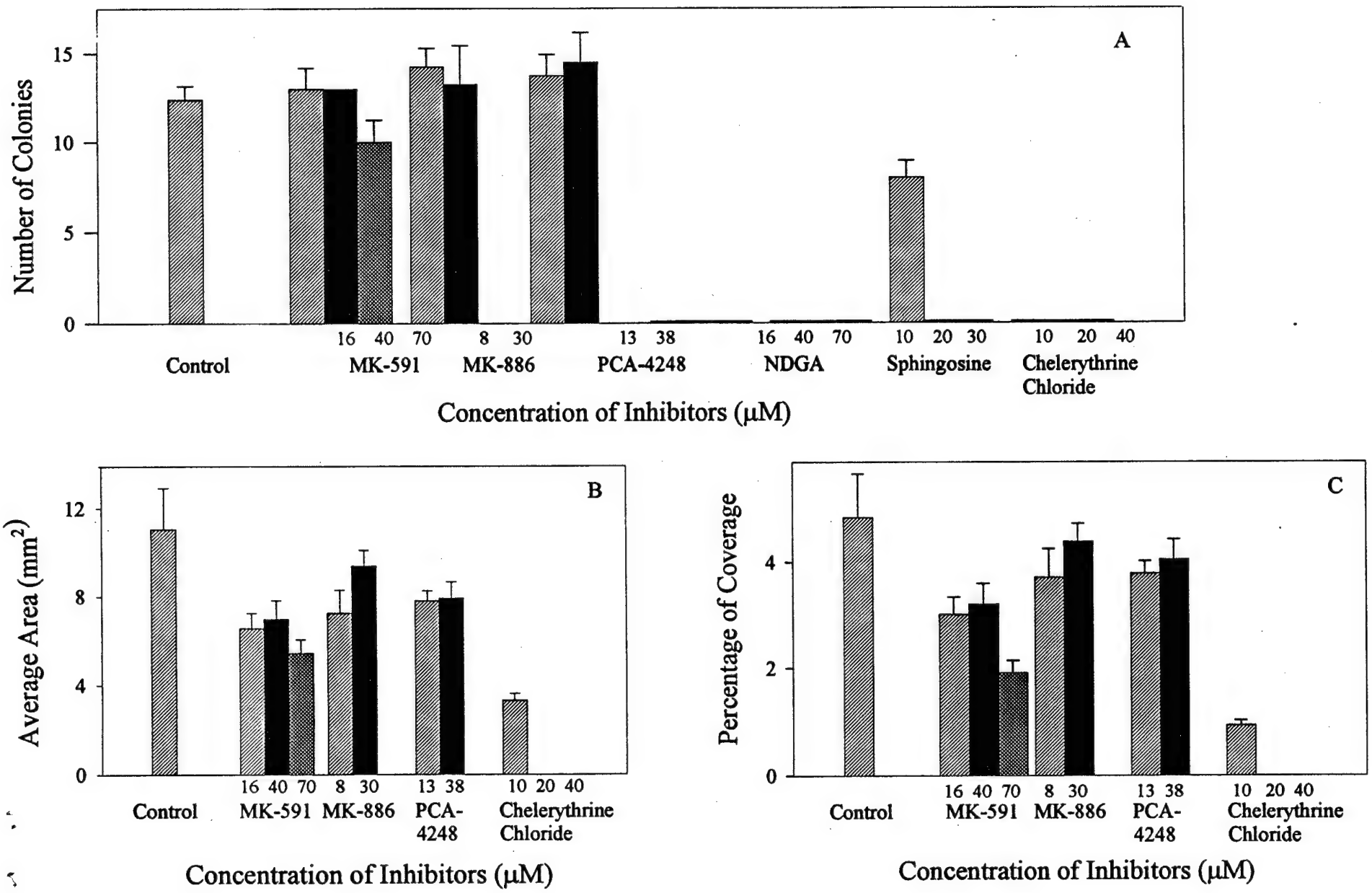
40
Concentration of Thapsigargin (μM)

Figure 3.


A
Number of Colonies
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8 30
13 38
16 40 70
10 20 30
10 20 40
Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)
B
Average Area (mm²)
0
4
8
12
16 40 70
8 30
13 38
10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)
C
Percentage of Coverage
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16 40 70
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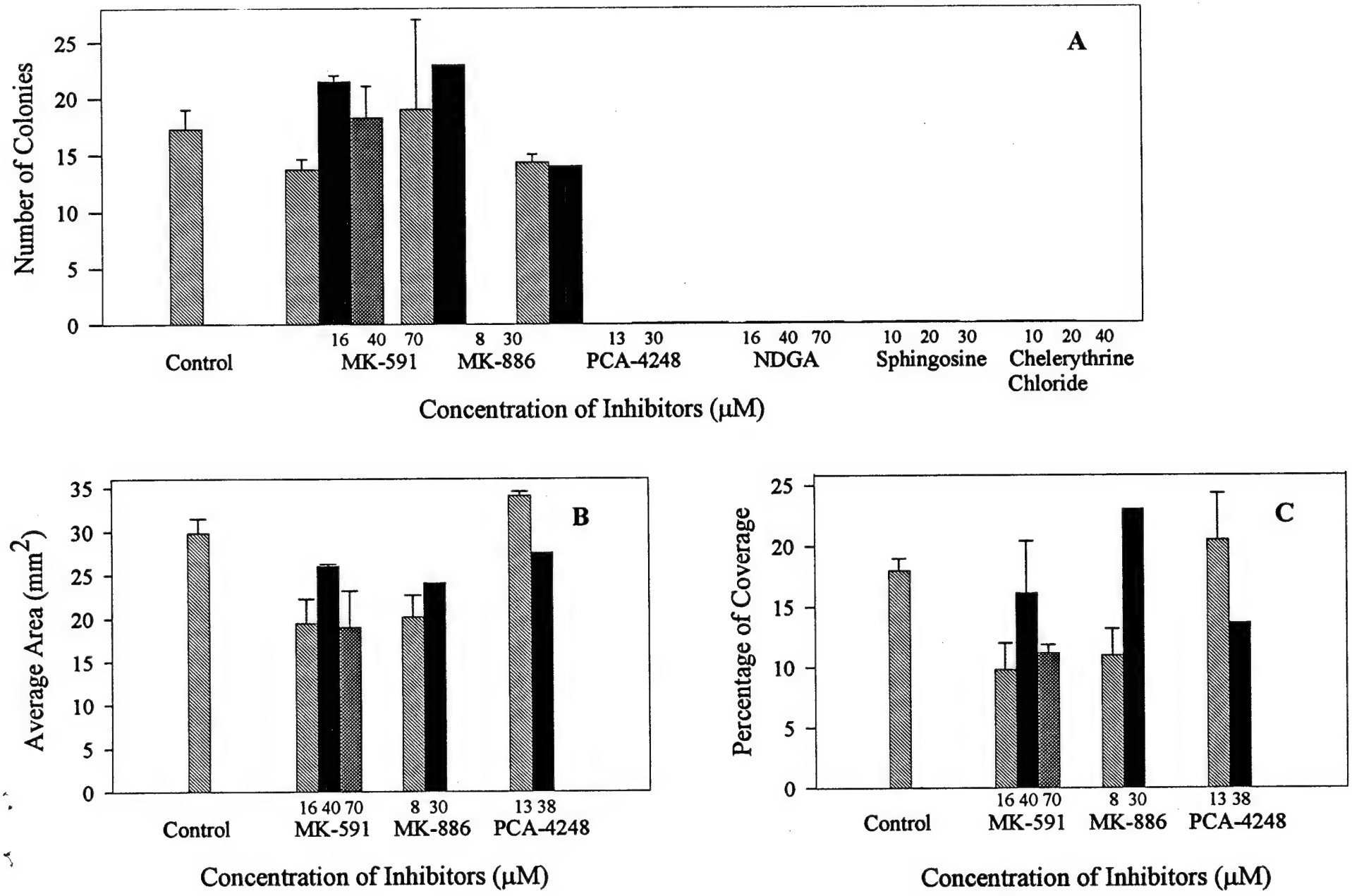
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

Figure 4.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Number of Colonies
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MK-886
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PCA-4248
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NDGA
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Sphingosine
10 20 40
Chelerythrine Chloride
Concentration of Inhibitors (μM)
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Average Area (mm2)
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MK-591
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MK-886
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PCA-4248
Concentration of Inhibitors (μM)
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Percentage of Coverage
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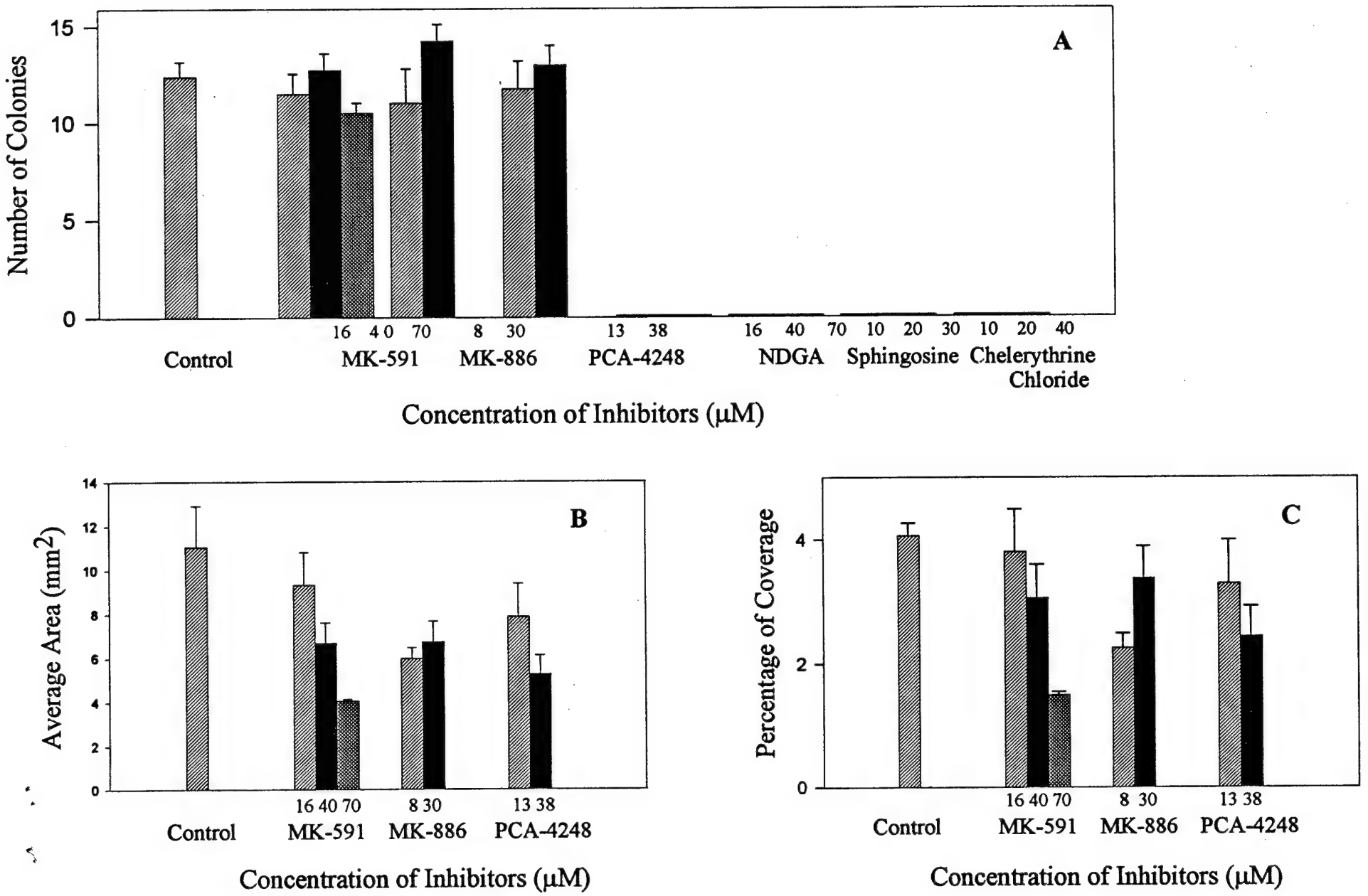
MK-591
8 30
MK-886
13 38
PCA-4248
Concentration of Inhibitors (μM)

Figure 5.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Number of Colonies
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Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)
B
Average Area (mm2)
14
12
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16 40 70 8 30 13 38
Control
MK-591
MK-886
PCA-4248
Concentration of Inhibitors (μM)
C
Percentage of Coverage
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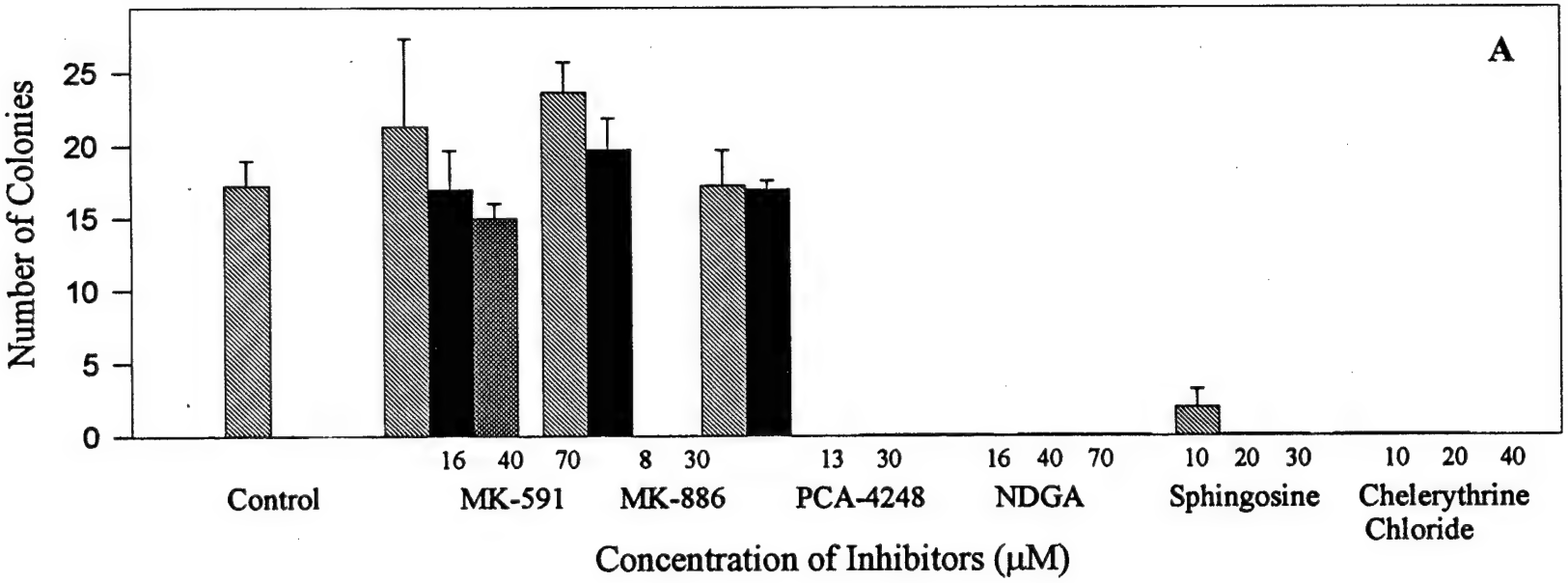
16 40 70 8 30 13 38
Control
MK-591
MK-886
PCA-4248
Concentration of Inhibitors (μM)

Figure 6.


A
Number of Colonies
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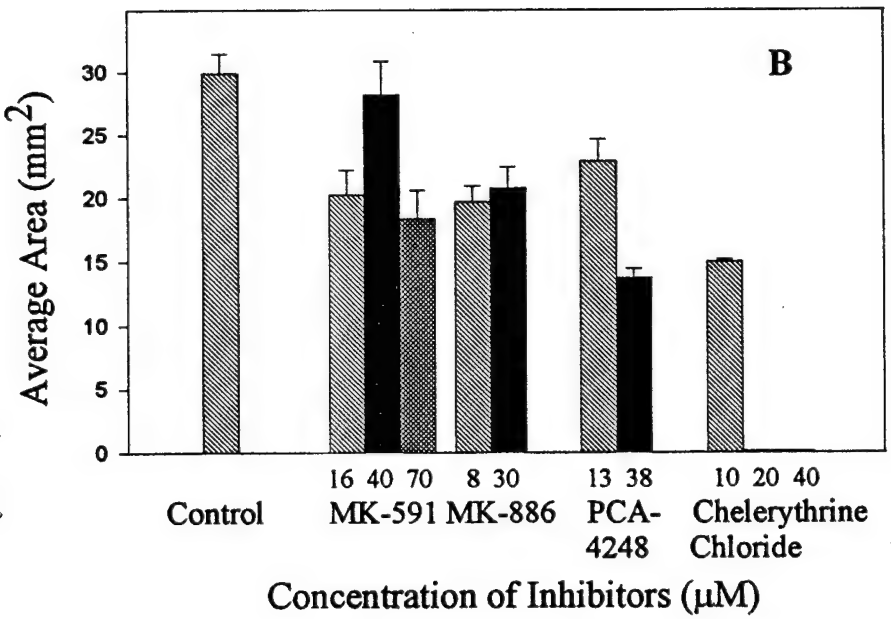
0
16 40 70
8 30
13 30
16 40 70
10 20 30
10 20 40
Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)


B
Average Area (mm2)
30
25
20
15
10
5
0
16 40 70
8 30
13 38
10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

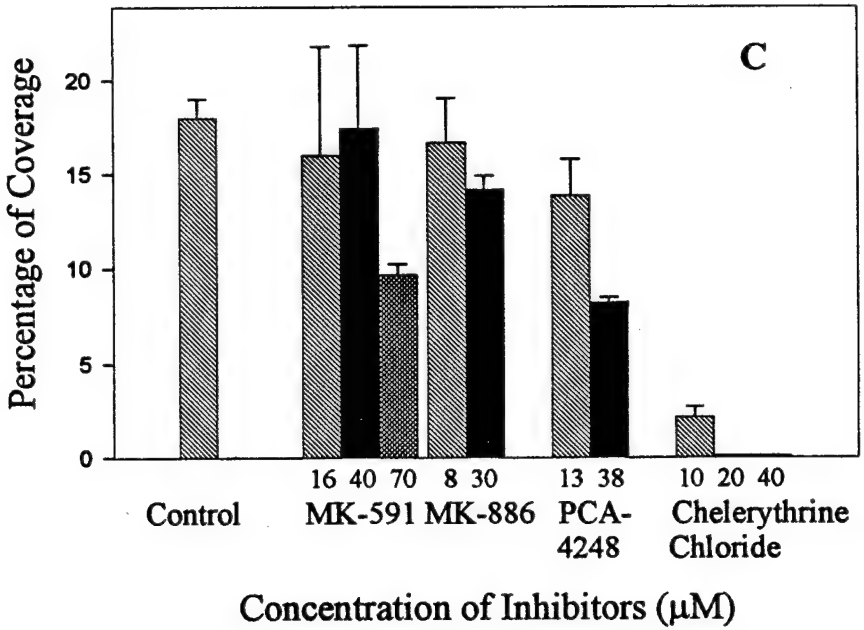
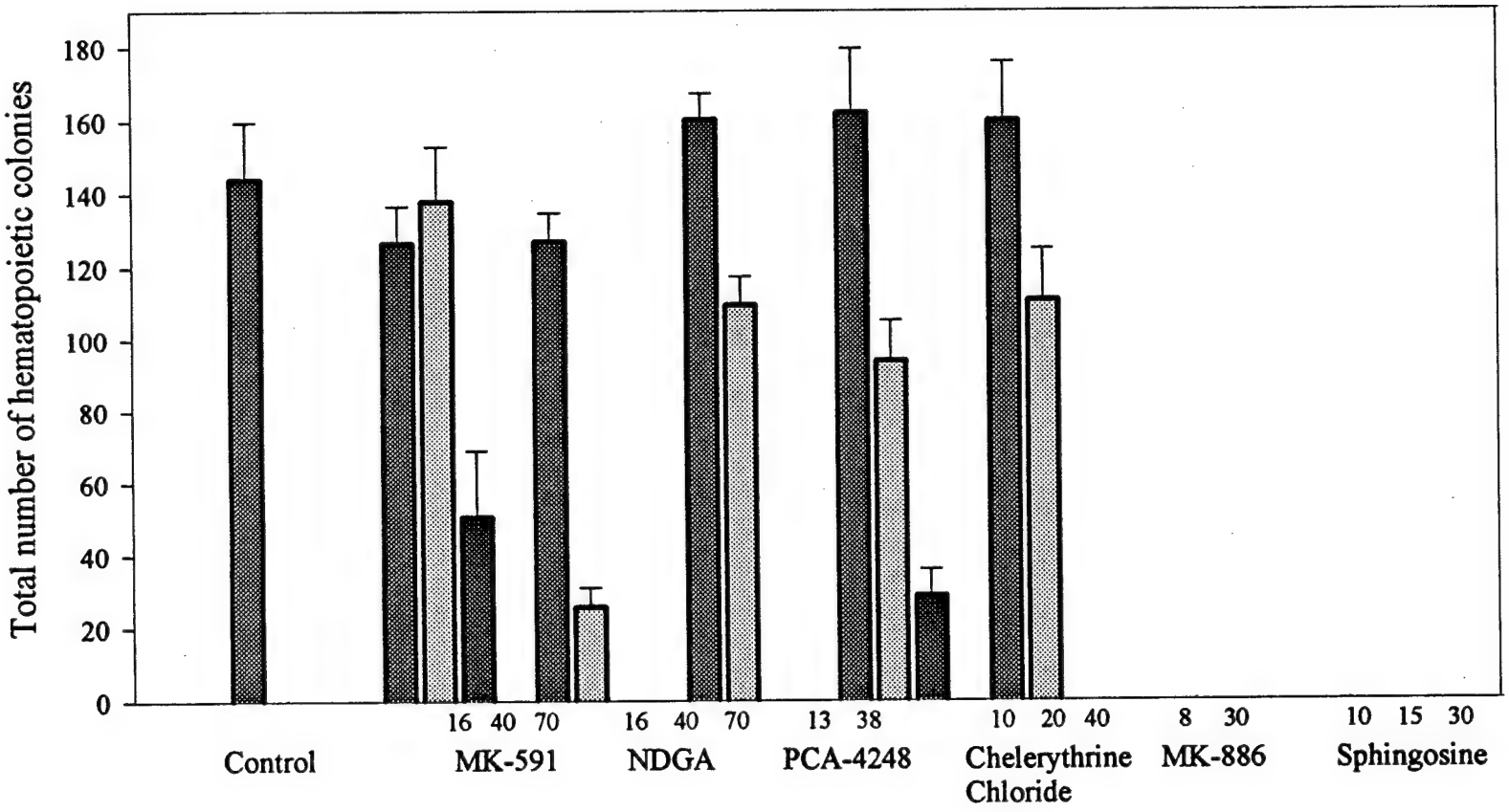

C
Percentage of Coverage
20
15
10
5
0
16 40 70
8 30
13 38
10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

Figure 7.



The treatment of human bone marrow cells with drugs at different concentrations (μM)

Figure 8.

CROSS TALK BETWEEN MAP KINASE PATHWAY AND ARACHIDONIC ACID PATHWAY IN THE SIGNALING CASCADE OF IGF-1 IN BREAST CANCER CELLS.

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ABSTRACT

Insulin Growth Factor I /(IGF-1) when bound to its receptor, initiates a molecular signaling cascade that ultimately results in cell growth. Two of the many pathways involved in the growth of breast cancer cells are the Arachidonic Acid /(AA) pathway and the Ras-MAPK pathway. The former involves the release of AA from the cell membrane in response to IGF-I. The cyclooxygenase pathway and the lipoxygenase/(LO) pathways produce eicosanoid metabolites which are known to stimulate the growth of cancer cells. The MAP pathway deals with autophosphorylation of the IGF-1 receptor and the subsequent activation of ras, raf, and ultimately MAP kinase; MAPK activates various transcription factors leading to cell division. In the present study, we have analyzed the role of raf, MEK, MAP Kinase and 5-LO in the signaling of IGF-1 in human breast cancer cells /(578T). Raf, MEK and MAP Kinase were phosphorylated within 1 minute of IGF-1 treatment, reaching its peak by five minutes. Tyrosine phosphorylation of 5-LO was observed within 1 minute of IGF-1 stimulation. These results suggest that both signaling pathways are activated in response to IGF-1 in human breast cancer cells. To resolve the exact role of each of these kinases and establish a possible cross talk between the signaling pathways, we used several known inhibitors of these pathway. By blocking each of the pathways at different points, we should be able to pinpoint the exact relationship between the two cellular signaling pathways. The results suggest that there is cross talk between the two signaling pathways. Further studies are under way to determine the exact relationship.

BACKGROUND:

Although screening mammography and the increased use of breast conserving surgery and adjuvant chemotherapy have improved the quality of life and prolonged survival for women with breast cancer, additional therapeutic strategies are needed to combat the disease. Various studies

have suggested dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids, particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Activation of AA metabolism is initiated by the release of AA from the phospholipid pool by the enzyme phospholipase A2 (Axelrod et al. 1988). AA is metabolized through the cyclooxygenase pathway which results in prostaglandins production (Boyle et al. 1994) or through the 5-lipoxygenase (5-LO) pathway, which results in the production of leukotriene (Henderson, 1994). Both prostaglandins and leukotriene directly stimulate the growth of malignant cells (Lee and Ip, 1992; Snyder et al. 1989). Agonist stimulation of cells initiates the translocation of enzymatically active 5-LO from a soluble site to a membrane bound site where it interacts with 5-LO activating protein (FLAP). FLAP is an 18 kDa membrane protein required in peripheral cells for the activation of 5-LO which results in the synthesis of the leukotriene LTB4 (Rouzer et al. 1990; Miller et al. 1990; Woods et al. 1995).

Compounds which preferentially inhibit AA metabolism are emerging as potentially important tools for cancer management. Although most experiments so far have focused on inhibitors of the cyclooxygenase pathway, there does not appear to be a direct correlation between the inhibition of cyclooxygenase activity and the inhibition of tumor growth (Fulton, 1984; Feldman and Hill, 1985; Noguchi and Ohta, 1993). Inhibitors of 5-LO metabolism have shown promise in the treatment of asthma, and shock (Larsen and Acosta, 1993; Henderson, 1994). Selective antagonists of 5-LO metabolism significantly reduce growth by a number of lung cancer cell lines (Avis et al. 1996). Linoleic acid stimulates tumor cell proliferation in vitro. In MDA MB-231 cells, inhibitors of the cyclooxygenase pathway and the lipoxygenase pathway were able to inhibit thymidine incorporation in these cells (Earashi et al. 1996). Recently it has been shown that proliferation of MCF-7 human breast cancer cells was blocked by specific inhibitors of bioactive lipids such as MK591, MK886 and AA861 (Zhang et al. 1996). In malignant transformed, prostate cells, a 5-LO inhibitor caused inhibition of proliferation indicated by decrease in DNA synthesis (Anderson et al. 1994). Tyrosine phosphorylation of 5-LO is important for its activation and translocation to the membrane fraction in HL-60 cells. Lepley et al. (1996) reported that tyrosine kinase inhibitors blocked the activation of 5-LO, its association with FLAP and subsequent formation of 5-HETE by A23187 stimulation of HL-60 cells. Several of the 5-LO inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis. The exact mechanism of action of these inhibitors is still not clear, especially the signal transduction pathways.

Multicellular organisms have developed highly efficient regulatory networks to control cell

proliferation. The mitogens interact with their receptors and induce activation of a series of kinases which ultimately lead to an increase in gene expression and growth. Many of these mitogens activate *ras* which binds GTP and in turn activates *raf* and MAP kinase kinase (MEK). A common pathway for many mitogens also includes activation of MAP kinase which in turn is known to activate a wide variety of target proteins including transcription factors which control gene expression (Cobb et al. 1991; Nishida and Gotah, 1993; Frost et al. 1994). Tyrosine kinase inhibitors have been shown to block the growth of breast cancer cells by affecting the *ras* -MAP kinase signaling pathway (Das and Vonderhaar, 1996, Clark et al. 1996). Somatostatin signal transduction through its receptor SSTR4 involves simultaneous stimulation of AA metabolism and MAP kinase phosphorylation cascade through PTX-sensitive G proteins (Bito et al. 1994). SSTR4 activates MAP kinase and induces phosphorylation of the 85 kDa cytosolic phospholipase A2 in a PTX sensitive manner (Sakanaka et al, 1994). In addition the coexpression of 5-LO and FLAP has been demonstrated in transmembrane signaling of somatostatin receptor in hippocampal neurons (Lammers et al 1996). These signal transduction pathways shared by different mitogens may provide an efficient approach to accomplish clinically significant control of breast cancer.

In the present study we analyzed the effect of IGF on the growth of normal and a transformed breast cell lines, in relation to its effect on 5-LO and FLAP. We used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects in breast cancer. To test whether FLAP is physiologically functional and involved in IGF modulation of breast cancer cells, we used the compound MK-886, a potent and selective FLAP inhibitor. Also the effect of these inhibitors, along with tyrosine kinase inhibitors on various target signaling molecules such as MAP kinase, raf and MEK along with 5-LO was studied. If we can block the signaling pathways of breast cancer cells, it may lead to arrest of their uncontrolled growth, which ultimately could be of clinical significance.

MATERIALS:

Cell lines: Hs 578Bst- a normal breast cell line, and Hs 578T- a tumor cell line derived from ductal carcinoma of breast will be utilized. Both are estrogen receptor negative and will be obtained from ATCC, Rockville, MD.

Inhibitors: MK886, MK591, AA861, Thapsagargin, Wortmannin, tyrphostin, herbimycin, genestein will be obtained from Biomol, PA and PD 098059 from Parke-Davis, MI.

Antibodies: Antibodies for MAP kinase, PI3 kinase, cyclin D, cyclin E, cdk4, cdk6 will be obtained from Transduction Laboratories, Lexington, Kentucky.

METHODS:

Cell Culture: Cells were maintained routinely in Dulbecco's modified Eagle's medium supplemented with serum and growth factors.

Extraction and Western Blot

Hs 578T Cells were obtained from ATCC, IGF-1 was obtained from Intergen. Inhibitors were obtained from Biomol and Merck Frost.

Human breast cancer cells, (Hs578T), were serum starved for 48 hours and were treated with Insulin, IGF-1 or inhibitors and incubated for five minutes. Cells were then washed with 1X PBS and homogenised in lysis buffer containing 20 mM Hepes, 40 mM beta-glycerophosphate, 10 mM EGTA, 1 mM DTT, 150 mM NaCl, 1% NP40, 0.5% Sodium Deoxycholate, 1mM Sodium Orthovanadate, 1mM PMSF, 10ug/ml Aprotinin, 10ug/ml Leupeptin. Equal amounts of protein was loaded on a 10% SDS-Gel or immunoprecipitated and then resolved on a gel. Western blots were probed with specific kinase antibody and was detected using the ECL detection kit.

Release of arachidonic acid: Arachidonic acid release will be measured as described previously (Bito et al. 1994). Cells will be prelabelled with 3H-arachidonic acid in serum free medium. The cells will be washed and after various treatments, aliquots of the medium will be tested for the release of radioactive AA.

5-LO assay: Cytosol and nuclear fractions was prepared from cells treated with growth factors and inhibitors. These cell fractions were subjected to immunoprecipitation with anti-5LO antibody (Merck-Frost, Canada), resolved on a SDS-gel and after transfer to nitrocellulose probed with phosphotyrosine antibody.

Reverse transcriptase - polymerase chain reaction (RT-PCR) analysis: Poly (A) mRNA was prepared from the treated normal and breast cancer cells using Trizol method. Reverse transcriptase reaction was performed and PCR carried out using specific primers according to the method of Dixon et al. 1990. PCR products were resolved on an agarose gel and the southern blot was probed for specific oligo probes for FLAP and 5-LO (Lammers et al. 1996).

MAP kinase assay: Cell will be treated with growth factors and/or inhibitors, extracted with buffer and assayed for enzyme activity using a MAP kinase specific substrate peptide according to the method of Das and Vonderhaar (1996).

In addition the proteins will be resolved by SDS-PAGE, transferred to nitrocellulose and probed with a specific antibody to MAP kinase.

RESULTS AND DISCUSSION:

Signal Transduction of Bioactive Lipids in Breast cancer is being investigated in Hs 578T cell line. We have shown in the tumor line IGF-1, a growth factor, activates both MAP kinase and

5-Lipoxygenase during signal transduction. We have assayed the enzyme activity by analysing the phosphorylation of these proteins on a Western Blot using specific antibodies against each kinase. The normal counter part of this breast cancer line Hs 578Bst was also examined. In Hs 578T breast cancer line we have shown that IGF-1 activates both the ras-MAPK and the 5-Lipoxygenase pathway for signal transduction.

a) TIME COURSE OF MAPK ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with Erk-2 antibody and detected with ECL kit. MAPK is also phosphorylated at the tyrosine residue of the enzyme where it reaches its peak within 5 mins of IGF-1 treatment.

b) TIME COURSE OF RAF-1 and MEK ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with raf-1 or MEK antibody and detected with ECL kit. Raf-1 and MEK-1 is also rapidly activated, within 1-5 mins. of IGF-1 treatment.

c) TIME COURSE OF 5-LO ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with 5-LO antibody and detected with ECL kit. 5-LO, the enzyme involved in the Arachidonic acid pathway is very rapidly activated, reaching its peak within 1-5 mins of IGF-1 exposure.

d) EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY IGF-1

Various inhibitors of these pathways are being investigated on the activation of these kinases. After serum starvation cells were preincubated with the inhibitors (MK 591, MK886, Curcumin,

NDGA) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The inhibitors of the 5-LO pathway were able to block the activation of MAPK by IGF-1.

e) EFFECT OF 5-LO INHIBITORS ON SHC ACTIVATION:

f) EFFECT OF RAS-MAPK INHIBITORS ON 5-LO ACTIVATION:

After serum starvation cells were preincubated with the inhibitors (FTase, PD98059 10μM. PD98059 50μM, LY294002) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The western blot was probed with 5-LO antibody.The inhibitors of the ras-MAPK pathway were not able to block the activation of 5-LO by IGF-1.

Thus the activation of 5-LO is upstream of activation of MAPK

f) EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY INSULIN

Cells were also treated with Insulin and similar study was carried out with these breast cancer cells. After serum starvation cells were preincubated with the inhibitors of AA pathway (MK886, PCA; Curcumin) for 30 min prior to treatment with insulin. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. Cells were able to activate MAPK in the presence of Insulin and this activation was blocked in when preincubated with 5-LO inhibitors.

g) RT-PCR of 5-LO IN THE PRESENCE OF INHIBITORS AND IGF-1:

These data suggest that in breast cancer cells, IGF which acts as a mitogen transduces its signal through multiple signaling pathways. This growth factor activates MAPK which is a well

known pathway for mitogenesis, however in the present study we show that both the AA pathway and the ras-MAPK pathway is activated in the presence of IGF-1. The inhibitors of the 5-LO pathway are able to completely block the IGF-1 induced stimulation of MAPK suggessting a possible cross talk between the two important signaling pathways. In the presence of MAPK inhibitors however the activation of 5-LO was not blocked indicating that 5-LO is upstream of MAPK signaling. We are analysing the AA metabolites currently in the presence of these various inhibitors which will give us a better understanding of the cross talk between the two pathways.

FIGURE LEGENDS:

FIG. 1. TIME COURSE OF MAPK ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with Erk-2 antibody and detected with ECL kit.

FIG. 2. TIME COURSE OF RAF-1 ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with raf-1 antibody and detected with ECL kit.

FIG. 3. TIME COURSE OF MEK ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with Mek-1 antibody and detected with ECL kit.

FIG. 4. TIME COURSE OF 5-LO ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with 5-LO antibody and detected with ECL kit.

FIG. 5. EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY IGF-1
After serum starvation cells were preincubated with the inhibitors (MK 591, MK886) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel.

FIG.6. EFFECT OF RAS-MAPK INHIBITORS ON 5-LO ACTIVATION BY IGF

After serum starvation cells were preincubated with the inhibitors (FTase, PD98059 10μM. PD98059 50μM, LY294002) for 30 min prior to treatment with IGF. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The western blot was probed with 5-LO antibody.

FIG. 7. EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY INSULIN
After serum starvation cells were preincubated with the inhibitors (MK886, PCA, Curcumin) for 30 min prior to treatment with insulin. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel.

Figure 1

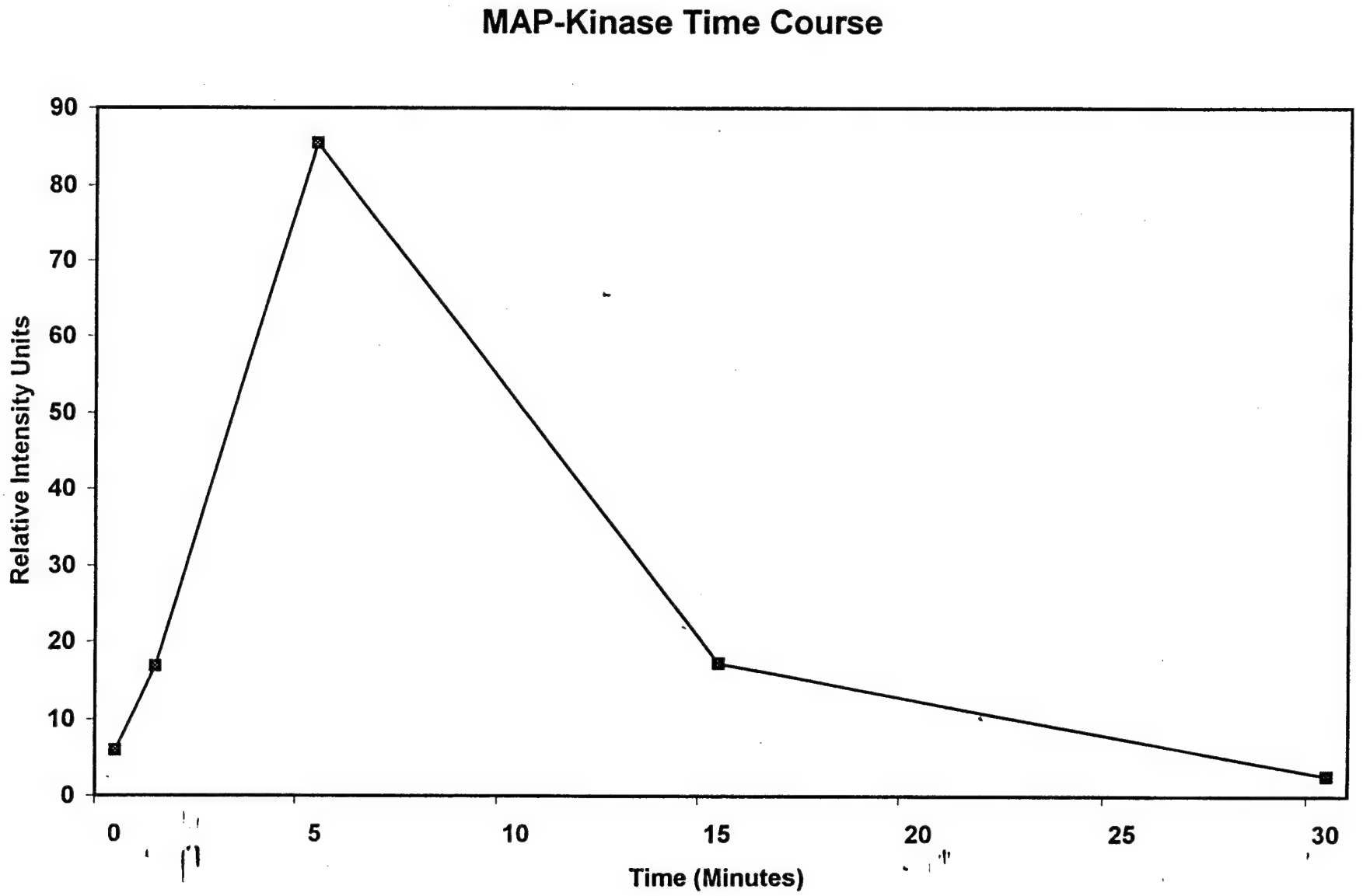
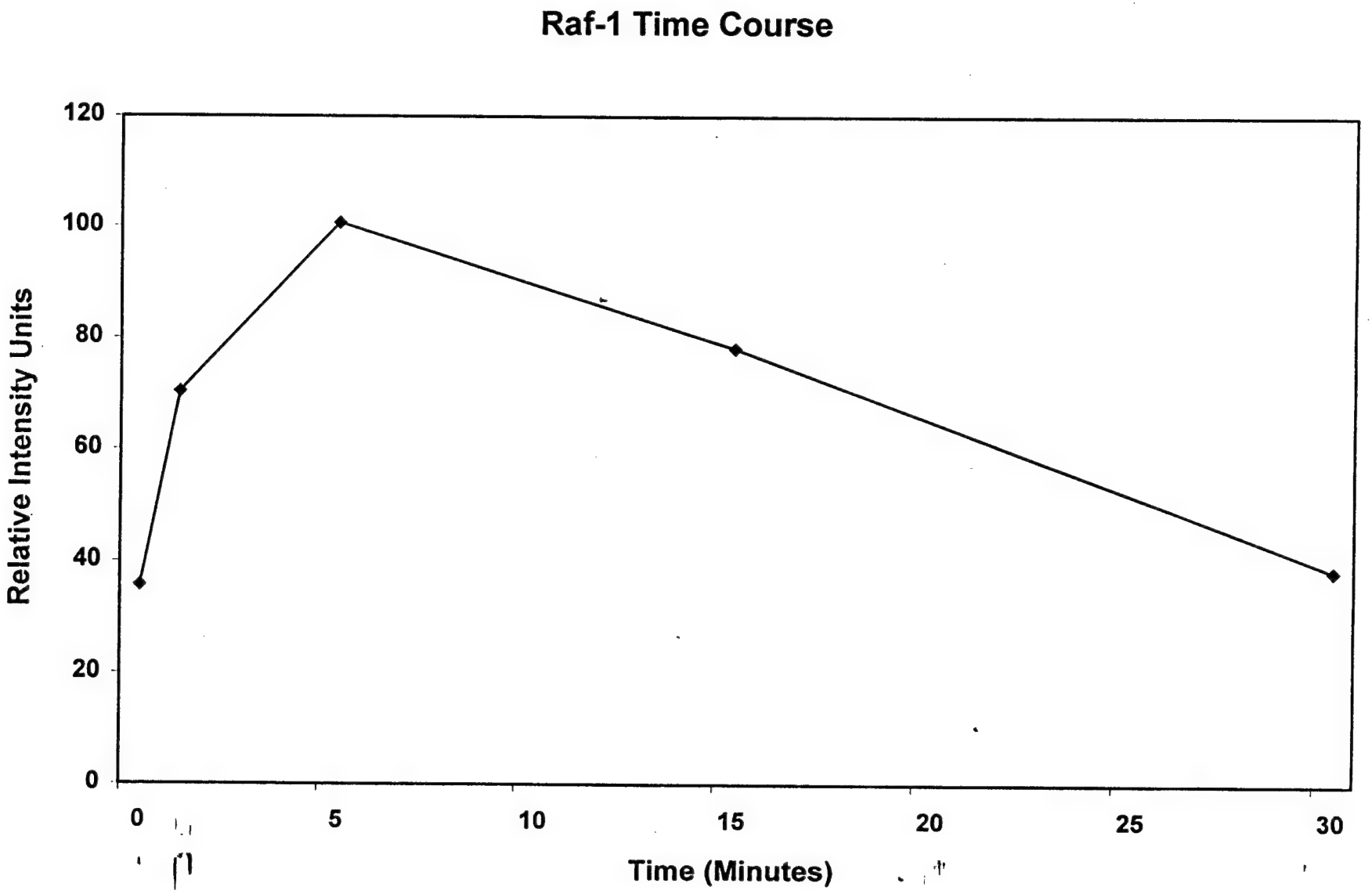

MAP-Kinase Time Course
Relative Intensity Units
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (Minutes)

Figure 2


Raf-1 Time Course
Relative Intensity Units
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100
80
60
40
20
0
0
5
10
15
20
25
30
Time (Minutes)

Figure 3

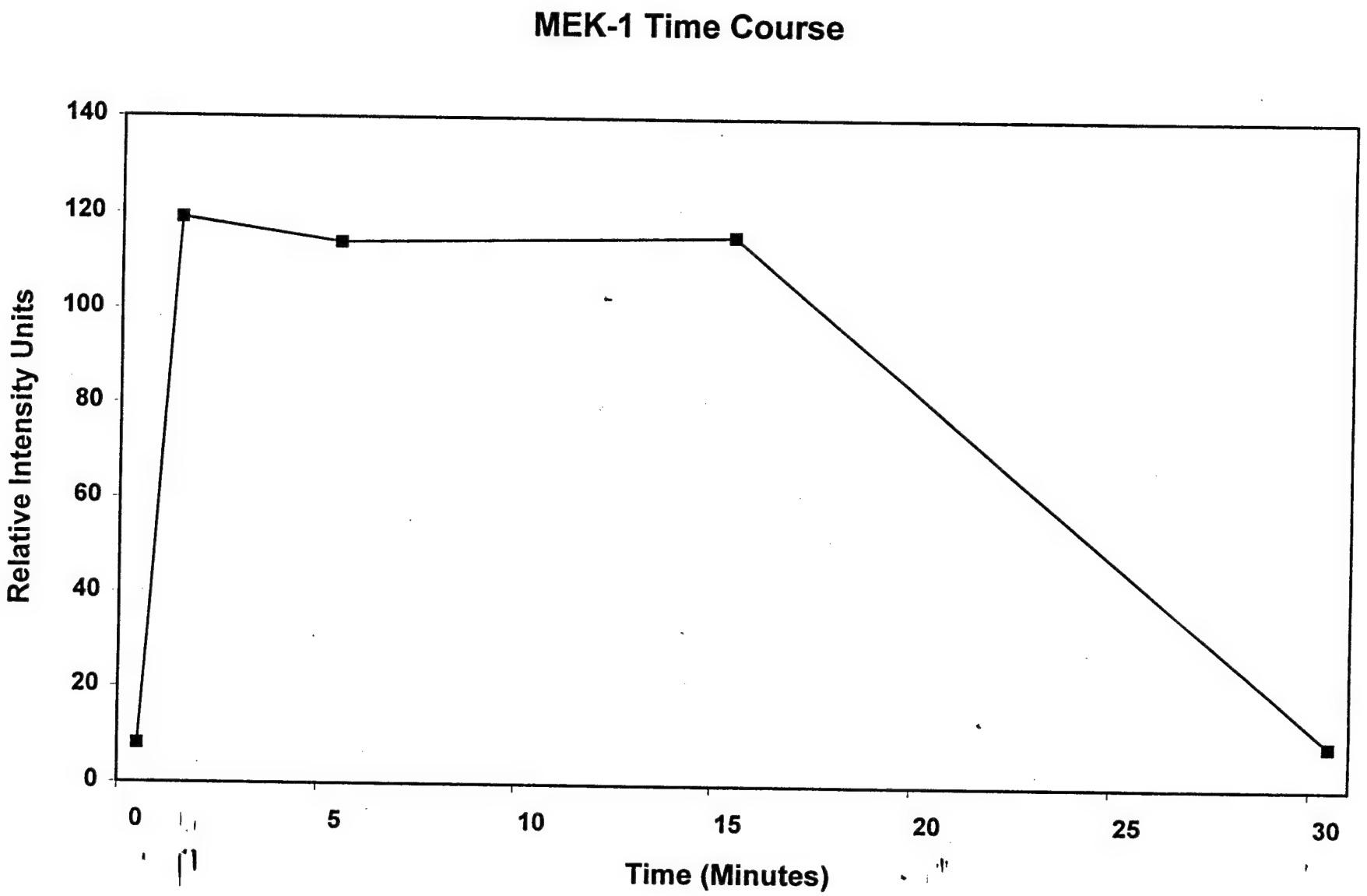

MEK-1 Time Course
Relative Intensity Units
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (Minutes)

Figure 4

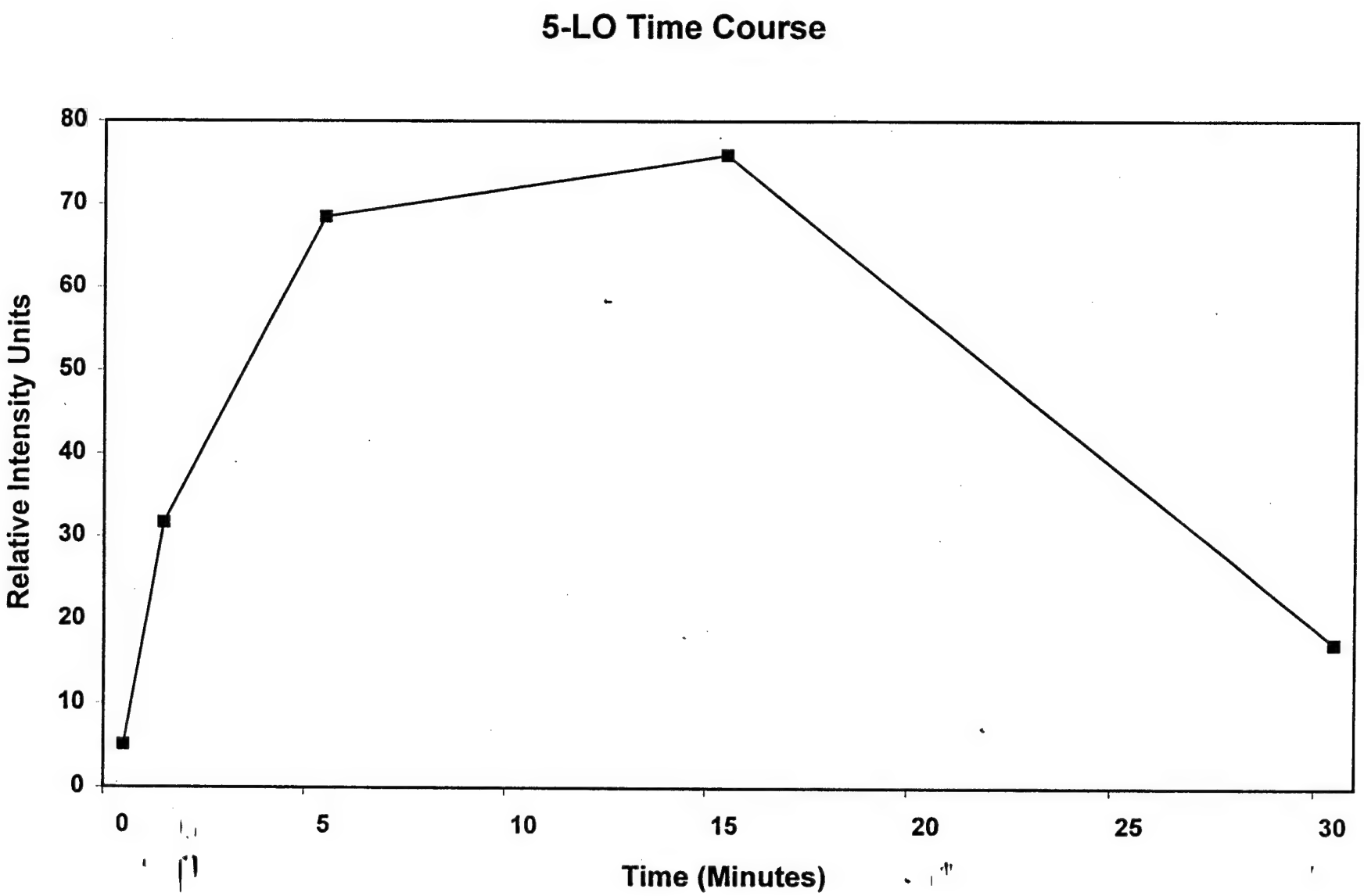

5-LO Time Course
Relative Intensity Units
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (Minutes)

Figure 5

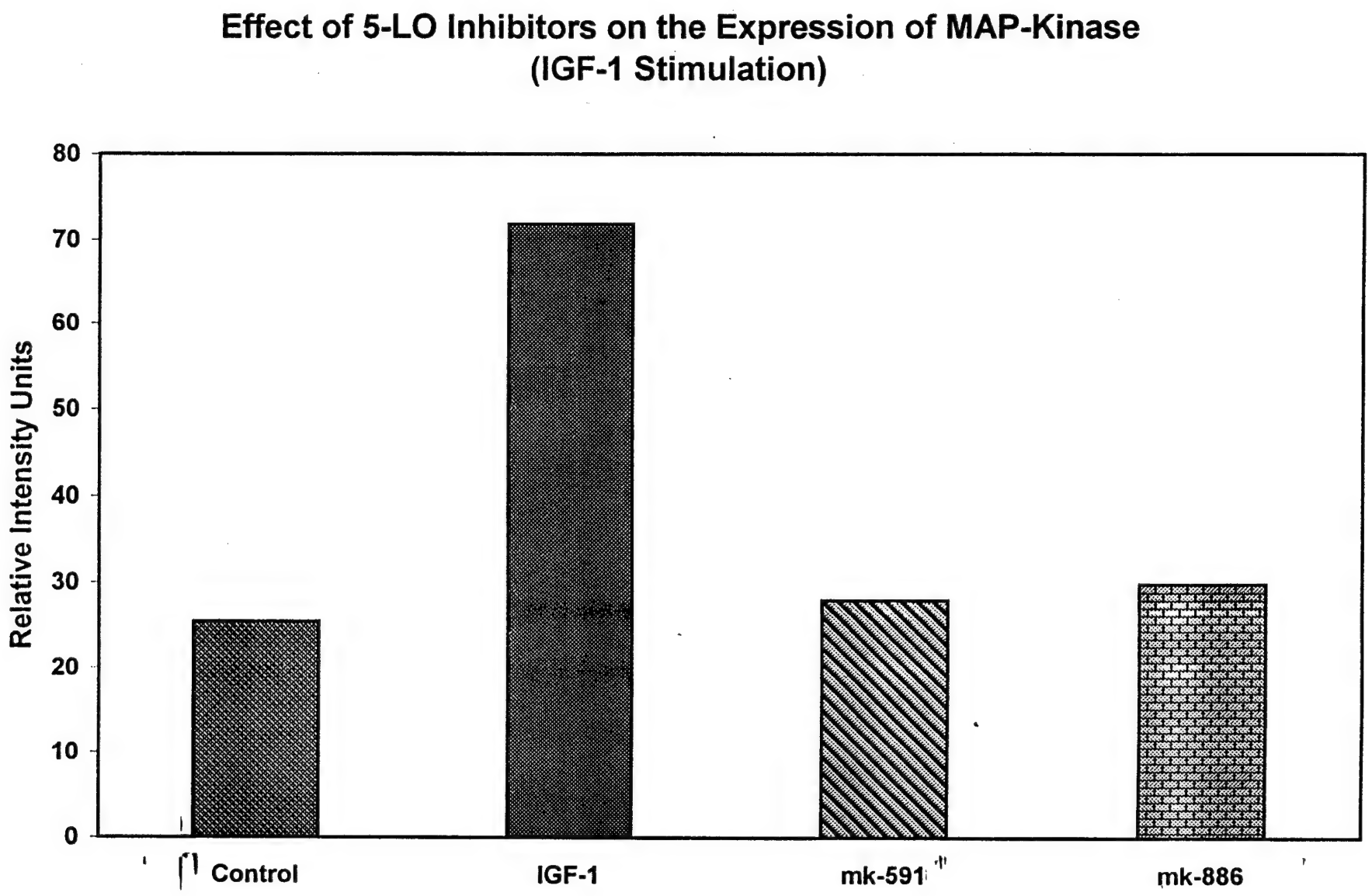

Effect of 5-LO Inhibitors on the Expression of MAP-Kinase
(IGF-1 Stimulation)
Relative Intensity Units
80
70
60
50
40
30
20
10
0
Control
IGF-1
mk-591
mk-886

Figure 6a

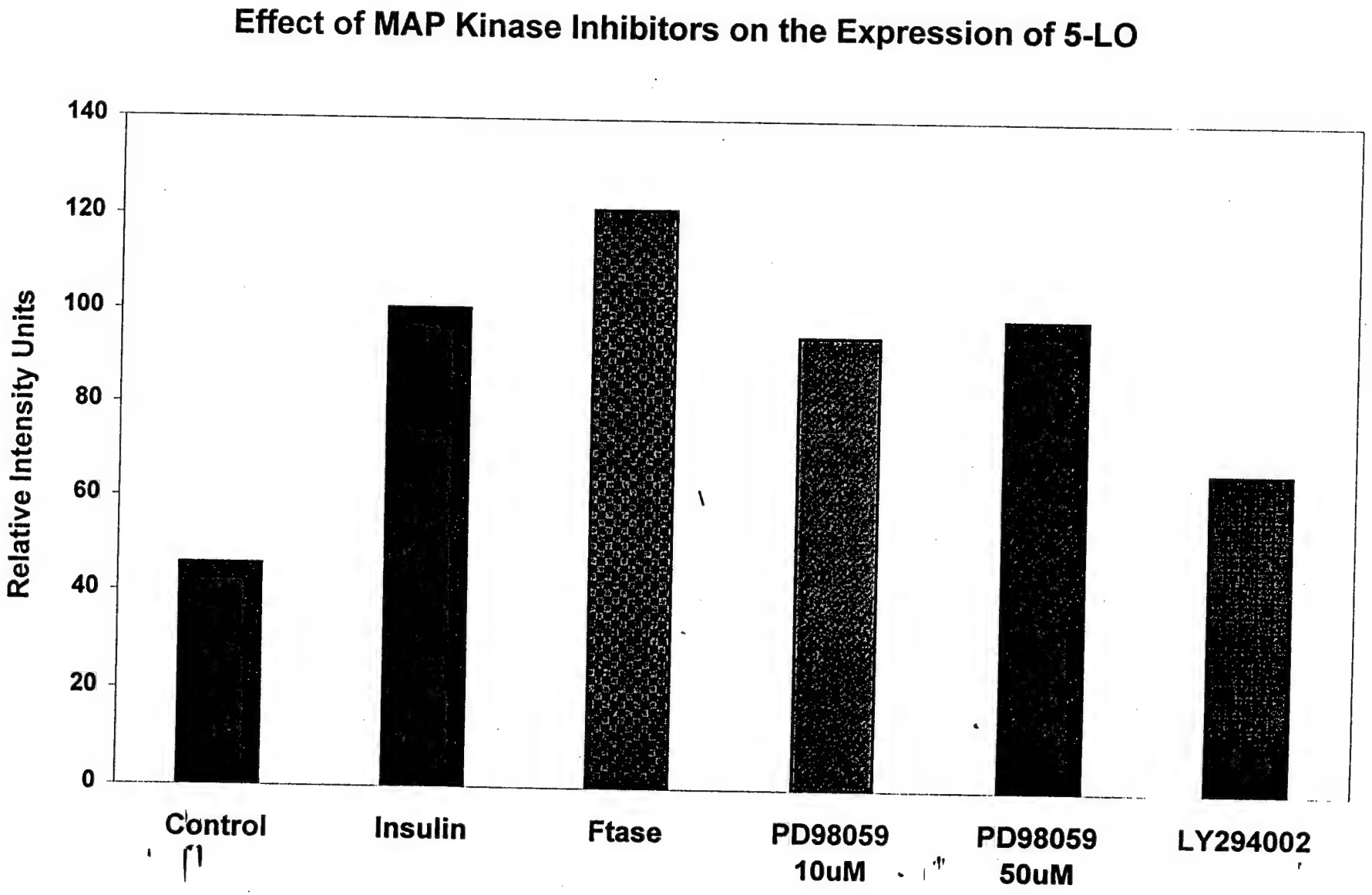

Effect of MAP Kinase Inhibitors on the Expression of 5-LO
Relative Intensity Units
140
120
100
80
60
40
20
0
Control
Insulin
Ftase
PD98059 10uM
PD98059 50uM
LY294002

Figure 6b

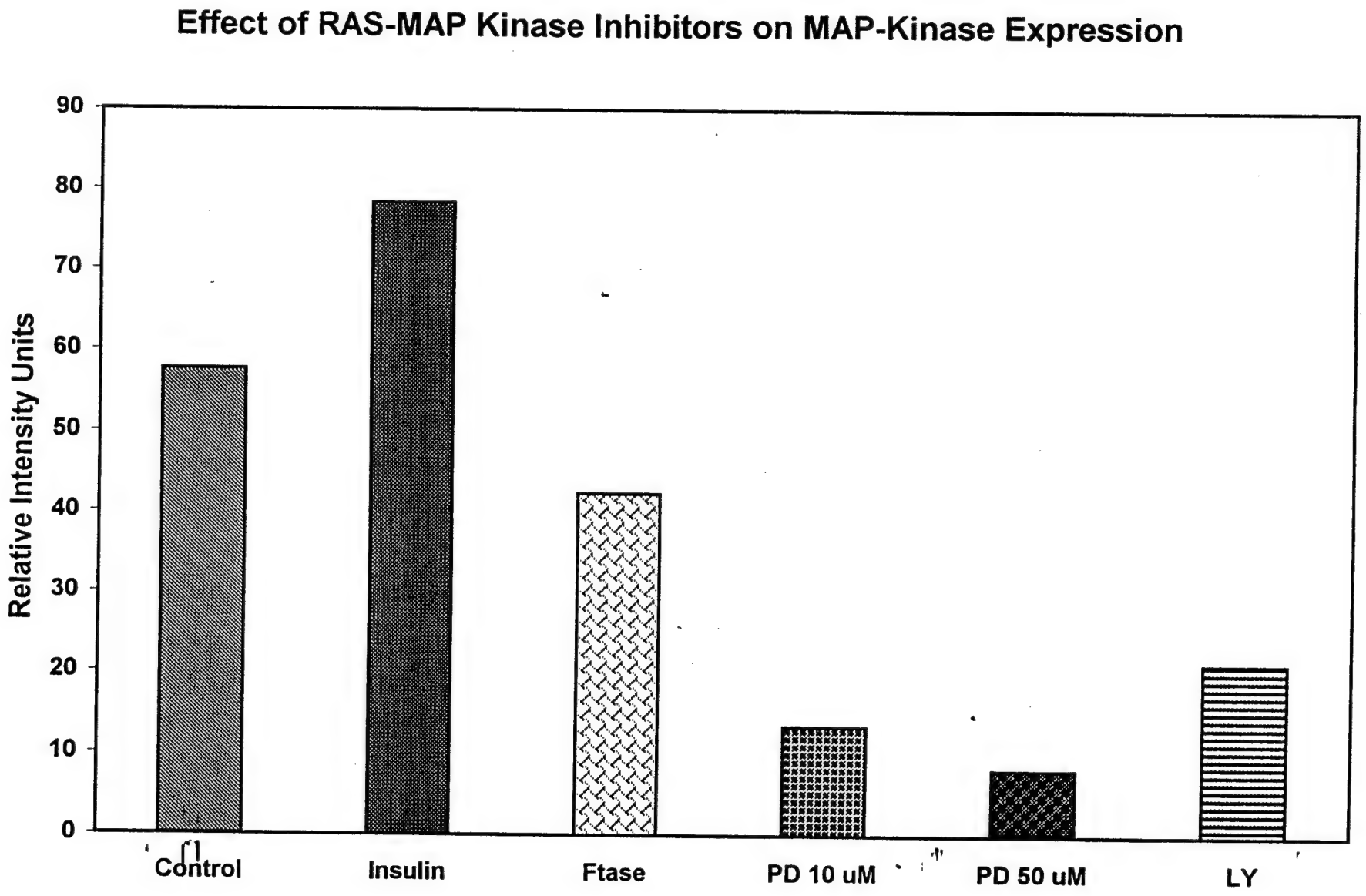
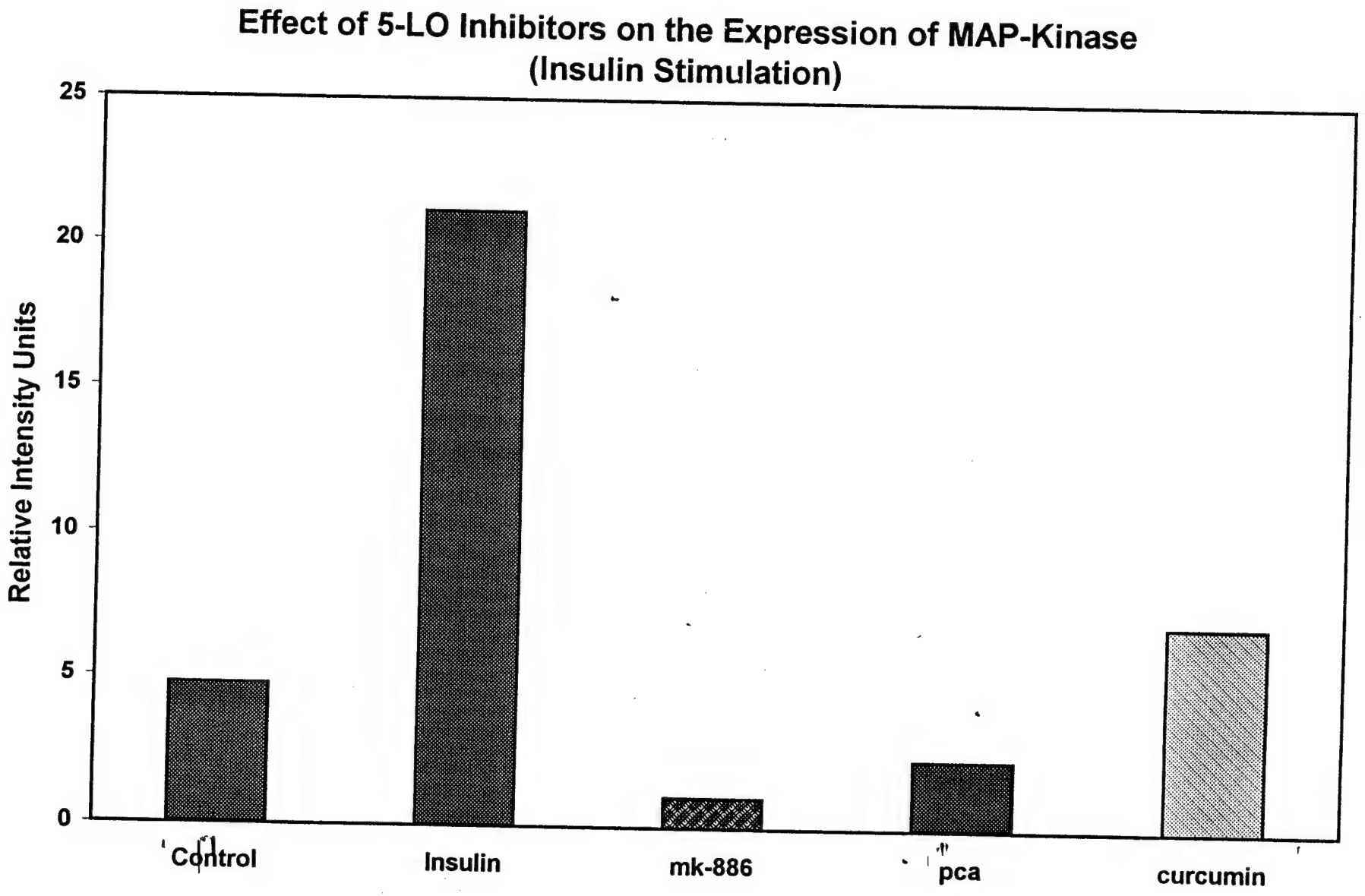

Effect of RAS-MAP Kinase Inhibitors on MAP-Kinase Expression
Relative Intensity Units
90
80
70
60
50
40
30
20
10
0
Control
Insulin
Ftase
PD 10 uM
PD 50 uM
LY

Figure 7


Effect of 5-LO Inhibitors on the Expression of MAP-Kinase
(Insulin Stimulation)
Relative Intensity Units
25
20
15
10
5
0
Control
Insulin
mk-886
pca
curcumin

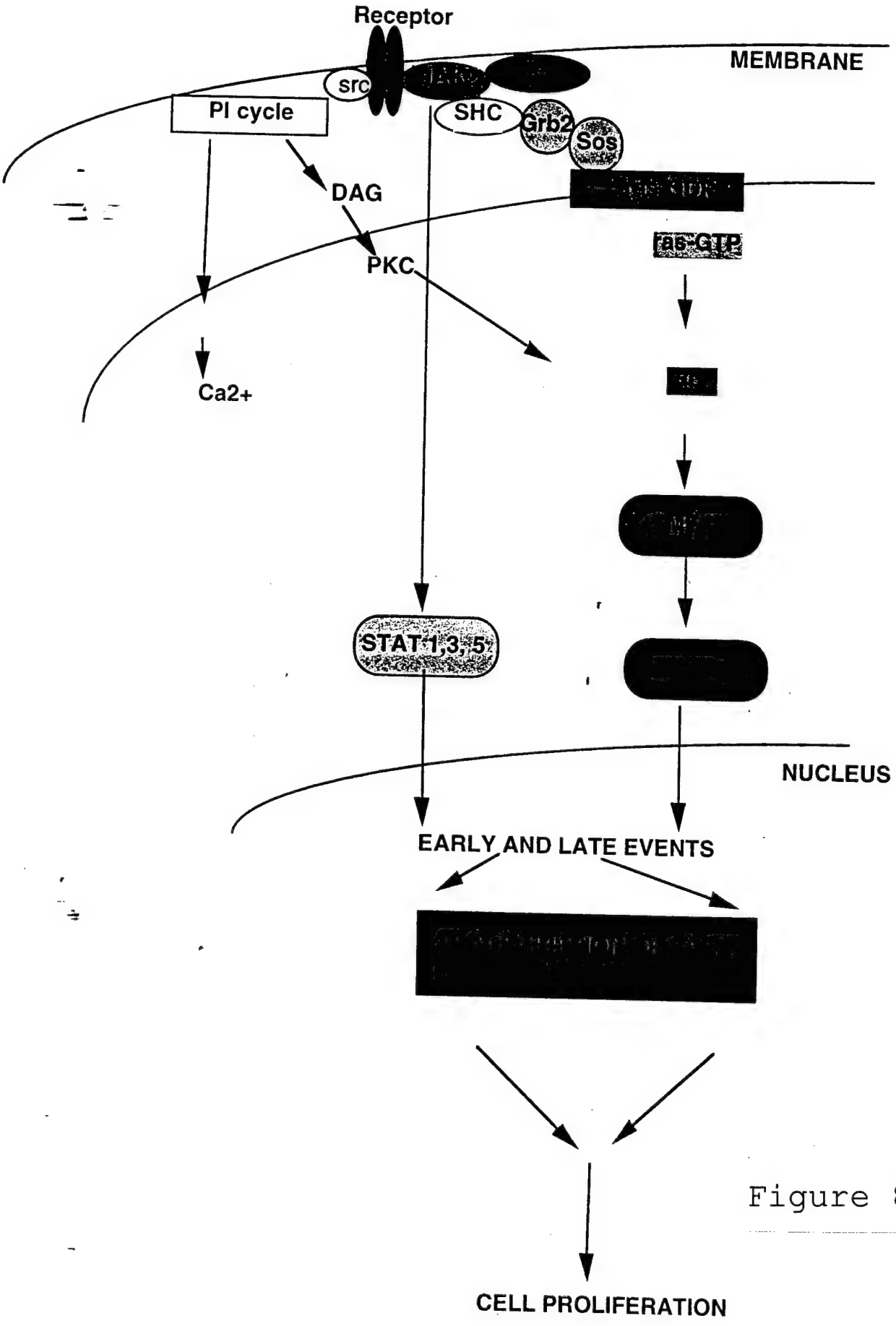

Receptor
MEMBRANE
src
SHC
Grb2
Sos
PI cycle
DAG
PKC
Ca2+
ras-GTP
STAT 1,3,5
NUCLEUS
EARLY AND LATE EVENTS
CELL PROLIFERATION

Figure 8

Treatment of MCF-7 breast cancer or human bone marrow cells with heteropolyanions

XiaoYan Zhang, Jonathan Leung, and Marti Jett. Walter Reed Army Institute of Research, Washington, DC 20307, and NCI, BMPB, Bethesda, MD.

Abstract: A group of compounds called heteropolyanions are free-radical scavengers that show excellent antiproliferative effects and do not induce drug-resistance as compared with doxorubicin. The IC_{50} of doxorubicin resistant MCF-7 cells are more sensitive to these heteropolyanions than normal WT MCF-7 cells. The treatment of synchronized ADR MCF-7 at G_1/G_0 with heteropolyanions causes cell death during DNA synthesis (S Phase). However, WT cell cultures start to die after 30 hours with the same treatment. Colony assays and advanced therapeutic studies with mice show that these compounds exhibit little toxicity to cultures of human bone marrow cells or mice, suggesting that they may be potentially useful as a form of human breast cancer therapy.

MATERIALS AND METHODS

Cells. The human breast cancer MCF-7 cells were obtained from ATCC. The cells were grown in Improved Modified Essential Medium (IMEM) containing 8% fetal bovine serum, 50,000 units/liter penicillin and 5,000 μg/L streptomycin at 37°C with 5% CO_2. Cells (0.5×10^6 cells/well with 8 mL IMEM in 4-well plates) were synchronized with thymidine (final concentration = 2mM) for 12 hours before they were treated with inhibitors in cell cycle studies.

Human bone marrow light density cells were obtained from Poietic Technologies Inc. The stroma colonies were grown in IMDM-based Long Term Culture Media[5] (LTCM) containing 25% horse serum (Hyclone) with 5% CO_2 and 100% humidity at 37°C.

Methyl(H^3)-thymidine incorporation: Inhibition of proliferation in WT or ADR MCF-7 cultures was performed in 96-well plates. Cells were plated 15,000 cells/well in 0.2 mL IMEM culture fluid[1] and incubated overnight. Inhibitors or heteropolyanions were added (50 µL) to achieve the indicated concentration and incubated for three days. During the last 18 hours, Methyl(H^3)-thymidine (1µCi / well) was added. The cells were trypsinized and harvested using Packard Unifilter System. Then, 40µL of Packard Microscint 0 scintillation cocktail was added and the filter plates were counted using Packard Top-count.

Sample preparation for cell cycle studies: Cells ($0.6x\ 10^6$) were plated in 4-well plates with 8 mL IMEM and incubated overnight. In order to synchronize the cells, 200 µL thymidine solution (80mM) was added to each well and incubated for 12 hours. After 12 hours, the media containing thymidine was aspirated and washed with FBS-free media twice and 8 mL of fresh media was added to each well. The inhibitors (using stock solutions that were more than 250 times the desired concentration) were added immediately in the fresh media and then incubated for different time periods. The cells were incubated with 1X trypsin, harvested, and then washed with 1X PBS and IMEM. The remaining cell pellets, to be used in DNA staining, were stored in a -80 C freezer.

DNA staining: Cells were stained as described[1]. 0.5 mL of NIM buffer containing 50µg/mL and 1µL RNase was added to approximately 0.35 µL cell suspension. The

stained samples were incubated in the dark at room temperature for at least 30 minutes. Using a 47 μm nylon mesh, the stained nuclear suspension was filtered.

Flow Cytometry and Data analysis: P.I. stained samples were measured on a FACScan flow cytometer equipped with Double-discriminator. The instrument was aligned as described.[1] Linar red fluorescence was collected using a Cell-fit program at 50-150 events.

Arachidonic acid metabolism: MCF-7 cells (0.3 million cells/well) were plated in 4-well plates with 7 mL IMEM and incubated for 24 hours. In order to starve the cells, the original media was removed and replaced with 7 mL FBS-free IMEM media and incubated for 48 hours. ^{3}H Arachidonic Acid (100 μCI/well) was added to each well and the plates were incubated overnight. Then the media was aspirated, washed with 10 mL 1X PBS, and aspirated once more. A time course experiment was performed for 2 minutes and 2 hours. Two control wells (IMEM + 2mM Ca^{+2} and IMEM + 2mM Ca^{+2} + 2 μg/mL IGF) were performed for each time period. The heteropolyanion drugs were premixed with the solutions mentioned above and added to the cells. After the desired time period was reached, 25 μL of 22M Formic Acid and 25 μL of 10mM BHT were added to stop the reaction. The plates were immediately placed on dry ice. The cells were scraped, washed with acidified water, and transferred to 15 mL tubes for extraction.

Arachidonic Acid Extraction:

Sep Pak Preparation: Sep paks were attached to the appropriate size syringe and placed in a vacuum filtration apparatus. The sep parks were prepared for extraction by adding 4 mL methanol followed by 10 mL distilled water and vacuuming each through.

Sample Preparation: The samples were centrifuged at 1000 g for 10 minutes. The supernatant was transferred to a new set of tubes and the pellet was stored on dry ice. Internal Standard tubes were made by adding 2 mL saline (original sample volume) to 13 mL acidified water. If needed, the pH of the samples should be adjusted to 3.5-4.0. Approximately 50 µL of internal standard (made by adding 5 mL methanol + 500 µL 15 HEDE + 50 µL Eicosatrienoic Acid) should be added to each sample to read 70,000-80,000 DPM. Two aliquots of 300 µL were removed from each sample and placed into scintillation tubes and counted (duel $^{3}H/^{14}C$).

Sample Extraction: The samples were poured through each sep pak, washed with 4 mL of 2% ethanol, and promptly removed to prevent air from getting through. The sep paks were washed with 10 mL of a 85% Acetonitrile/15% Methanol solution and the solutions were collected in 15 mL polypropylene tubes. Then, the sep paks were washed with 10 mL of a 100% Methanol solution and the solutions were collected into their corresponding tubes.

HPLC Preparation: All of the tubes that were collected from the Acetonitrile phase were dried using the Speed Vac (-80 deg C), washed with methanol and dried again. 100 µL of methanol was added to each tube and the solution was transferred to HPLC vials for HPLC analysis.

Colony Assay: Human bone marrow stroma colony studies were performed using light-density marrow cells[2]. Cells ($2x10^5$ cells/well) were plated in 4-well plates with 5 mL LTCM and drugs were added (according to their desired concentration) on the second day. The media was changed with fresh media either with (continuous treatment) or without drugs (pulse) every week. The stroma colonies were stained on the second and fourth week of treatment with HEMA 3 (differential hematology stain) and the number and size of the colonies were measured.

The hematopoietic progenitor colony assay was performed using Methylcellulose-based Colony Cocktail from Stem Cell Technologies, Inc. (HCC-4434). Small volumes of drugs (20-50μL) were premixed with the cocktail (4.5 mL) and 0.5 mL of $7.0x10^5$ cells/mL were added. The cells in the cocktail (either with or without drugs) were plated in a 35mm diameter gridded tissue culture plate (Nunc. Inc.). After a two week incubation period, the hematopoietic colonies were counted using an inverted phase microscope with 40X magnification. Using the standard criteria from Stem Cell Technologies Inc. (Atlas of Human Hematopoietic Colonies), the colonies were classified into categories that included: CFU-GM, BFU-E , CFU-E, CFU-Mix, and CFU-GEMM.

Both stroma colony assay and hematopoietic colony assay experiments were performed with four replicates and repeated three times with different lots of cells. Regardless of the lot of the cells, the same results occurred.

Preliminary injections were given to groups of mice to determine useful dosage for experimentation. The doses used were .05mg/mouse, .2mg/mouse, .3mg/mouse, .4mg/mouse, .5mg/mouse, .6mg/mouse, and .7mg/mouse.

a) Dose response of mice to Sm-HPA: Different amount of Sm-HPA was injected into 5 mouse in each group. The injection with 5.5mg or 3mg in 1mL for each mouse causes mouse death after 4 or 5 day injection. For the injection with 1.8mg or 1mg in 0.5 mL for each mouse, the former causes mouse death after two week injection and the later only showed hair lose after one week.

b) Treatment with chronic doses for therapeutic window: Ten groups of 7 mice were injected with Sm-HPA in 0.25 mL 0f 1:1 injection water / vingeral lactose injection fluid. The number of injections and the behavior of the mice were shown in Table 4. The average weights during the injection time period was shown in Figure 4.

c) Tungsten distribution in mouse organs: Mice were injected 2mg/mouse and necropsied at 2h, 4h, 6h, 8h, 16h, 24h, and 48h. The separate organs were digested and the measurement of W were shown in Table 5. In order to examine the digestion of W, the organs were collected at three different treatment with 2.1mg of Sm-HPA a) Inject 0.7mg/mouse each week for three weeks; b) Inject 0.7mg/mouse each week for three weeks and keep the mice for 7 more weeks; c) Inject 0.2mg/ mouse each week for ten weeks.

d) Pathology observation:

CONCLUSIONS

Observations and Results

In order to determine the amount of heteropolyanions that could be injected over a period of several weeks, preliminary injections were given to individual mice. The first mice was given 5.5mg and died on the fourth day. The second mouse, given 3mg, also died on the fourth day. The third mouse was given two injections of 1.8mg each, a week apart, and

died after the second injection. The fourth mouse was given a 1mg injection. Some hair loss occurred, though no other changes were observed.

The Observations of the experimental groups are tabulated in Table X.

After one injection, differences in the skin tightness of mice injected with high amounts of the heteropolyanions was immediately apparent. The .3mg/mice group had slightly tighter skin while the groups which received injections of heteropolyanions between .4mg/mice and .7mg/mice had significantly tighter skin. After three weeks, injections were discontinued in the groups receiving between .5mg/mice and .7mg/mice as the skin became too tight. Skin in groups receiving lower amounts of heteropolyanions tightened more gradually. Skin tended to loosen once injections were discontinued.

Significant hair loss began to occur in the groups receiving high amounts of heteropolyanions during weeks 4 through 7, after injections had ceased. The .7mg/mice group lost as much as ½ to ¼ of total body hair and the .6mg/mice group lost approximately ½ total body hair. Some hair recovery was later observed. Slight hair loss also occurred in the .4mg/mice group during weeks 6 and 7 and in the .3mg/mice group during weeks 8 through 11.

The average weight of the mice in the control and experimental groups is shown in Figure Y. For groups receiving low amounts of heteropolyanions, normal weight gain occurred for the first few weeks, with minor weight loss later on. In the .4mg/mice group, the weight loss was significant. With groups receiving larger injections, a dramatic weight loss was observed between weeks 3 and 4, with most weight regained after injections were discontinued.

CONCLUSIONS

1. The treatment of MCF-7 breast cancer or human bone marrow cells with

heteropolyanions

2. Proliferation data: 1 figure and 1 table; (Drug resistant test)

3. Colony assay

4. Cell cycle data: a) synchronized at G1 phase; b) synchronized at G2 phase.

5. Arachidonate metabolism.

6. Mice experiment.

Two groups of mice were given .7mg/mouse injections. One group was euthanized when injection was discontinued after the third week. The second was euthanized after ten weeks. Both groups were (necropsied?) and the concentrations of heteropolyanions measured in different parts of the body. Other groups were also (necropsied?) after ten weeks.

The .4mg/mouse group and the .5mg/mouse group were not euthanized nor given injections for a period of two months (?). After two months, injections were again attempted; however, the skin was too tight to administer injections, so heteropolyanions were administered orally.

a) Toleration of heteropolyanion for single dose.

RESULTS AND DISCUSIONS

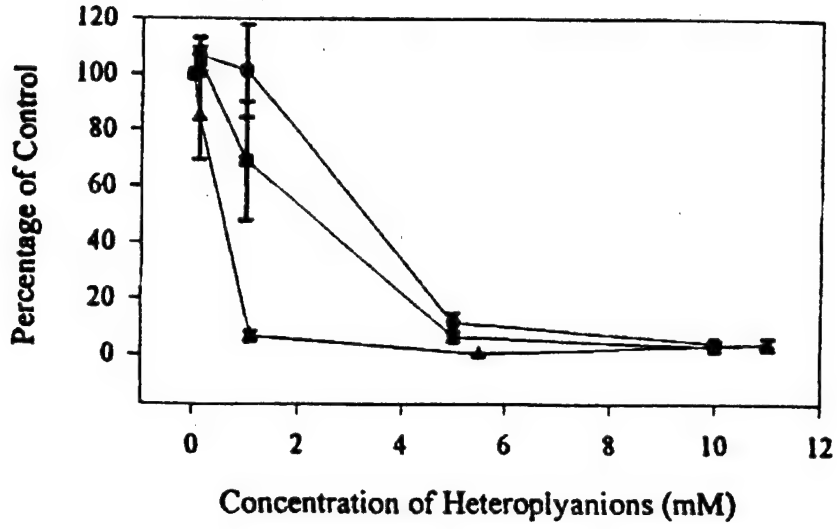
Inhibition of Proliferation were examined with heteropolyanions of group A, B, and C (Figure 1) in WT or ADR MCF-7 Cultures. Group A, [P5 W30 XO110]-m (X=) showed similar effective antitumor activity despite different charges. The typical data were shown in Figure 2 and the rest of them were shown in Table 1. It shows clearly that ADR MCF-7 cell cultures are more sensitive to these HPA than WT MCF-7. Almost all of IC50 for ADR cells are lower than these for WT cells. The group A polyanions The groups B and C do not show significant antitumor activity under present conditions (concentration 1-60μM). This may indicate that the size or the shape of the heteropolyanion are key factors in regards to biological activities.

The toxicity studies of the heteropolyanions to human bone marrow cells

Hematopoitic colonies of human bone marrow monnulear cells were treated with different concentrations of [SmP5 W30 O110]12- and [NaP5 W30 O110]14- to test for toxicity. The total number of colonies remaining after treatment are shown in Figures 3-6. The heteropolyanions were used in concentrations of 1mM, 6 mM, and 12 mM. As the concentration increases the total of the colonies decrease. The total of the colonies remain as much as 1/3 of control even at very high concentration of HPA (12μM).

The distribution of deferent colonies are tabulated in Table Y. The toxicity of the heteropolyanions was evident in the BFU-E and CFU-E colonies at 6μM. Both heteropolyanins did not have much effect on the CFU-GM colonies. All colonies of BFU-E and CFU-E were diminished and that of CFU-CM was slightly decreased at 12 mM.

Mice experiment:


Percentage of Control
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100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration of Heteroplyanions (mM)

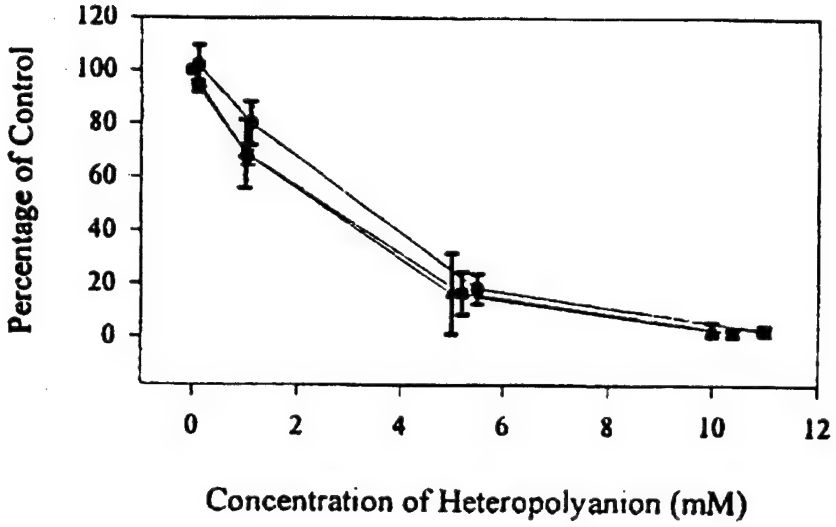

Percentage of Control
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration of Heteropolyanion (mM)

Figure 1.

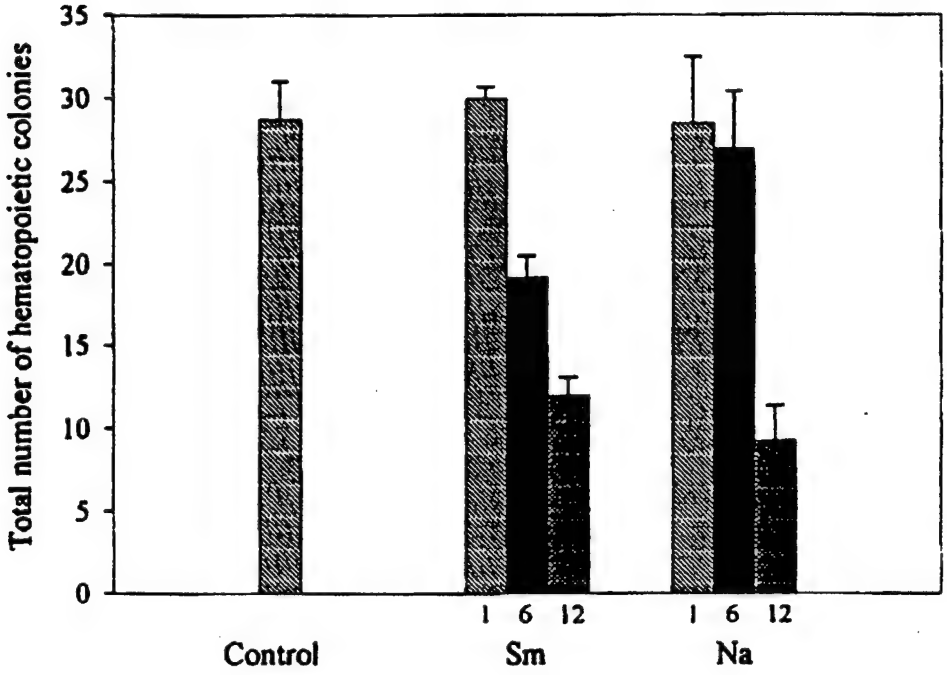
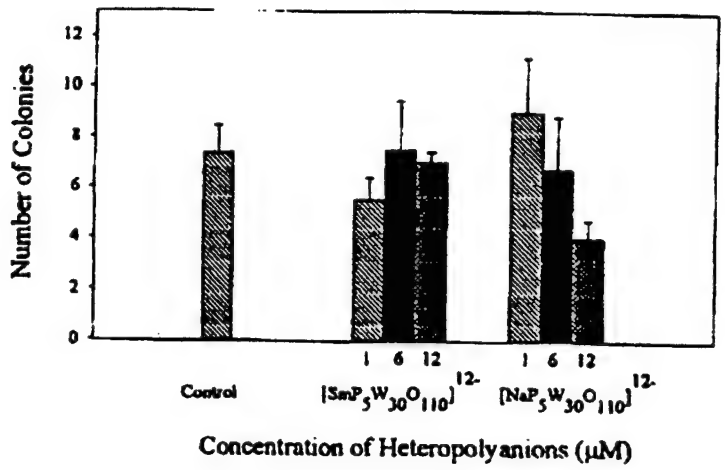
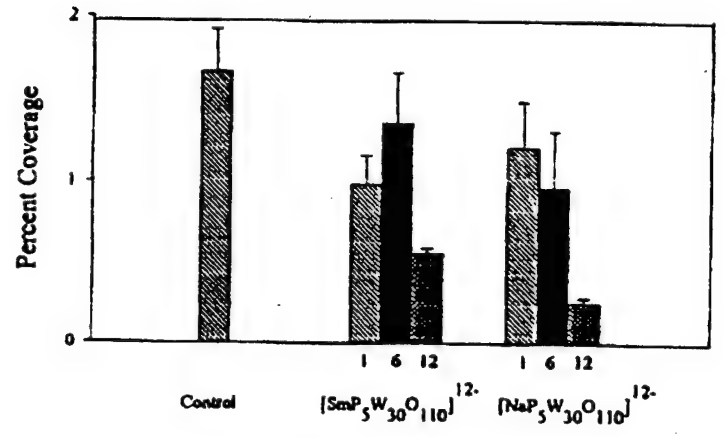

Total number of hematopoietic colonies
35
30
25
20
15
10
5
0
1 6 12
1 6 12
Control
Sm
Na

Figure 2.





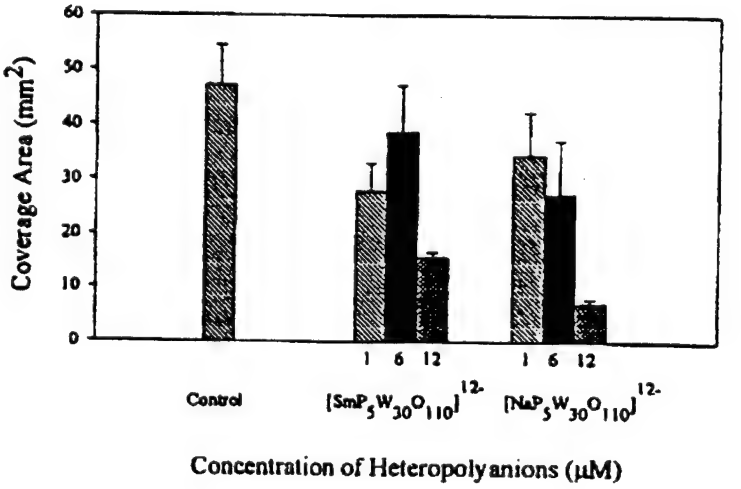
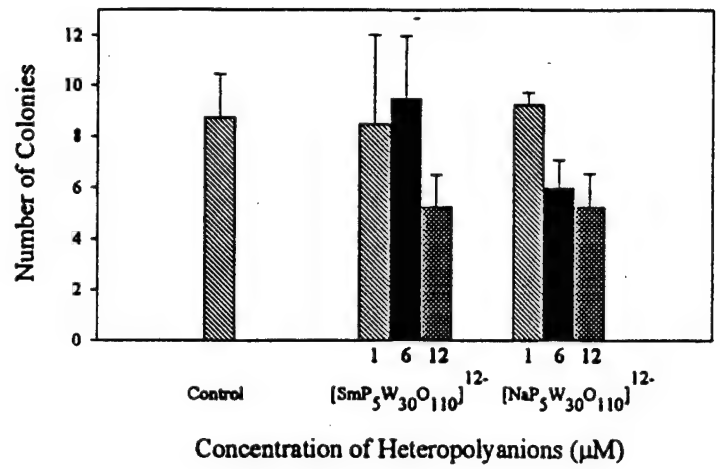
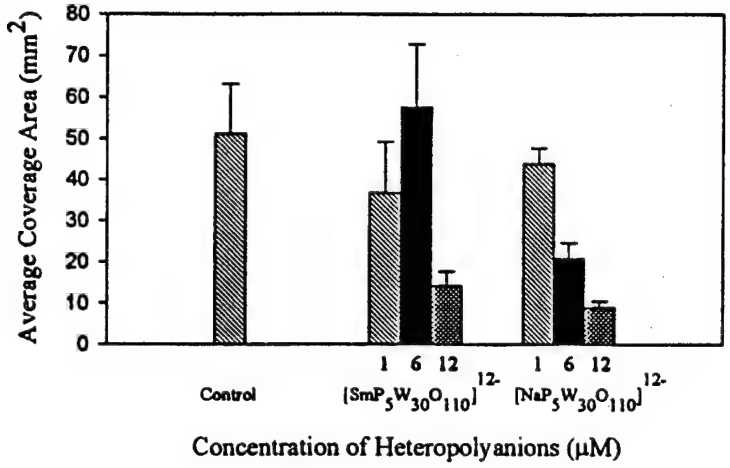


Figure 3. 2 weeks continuous





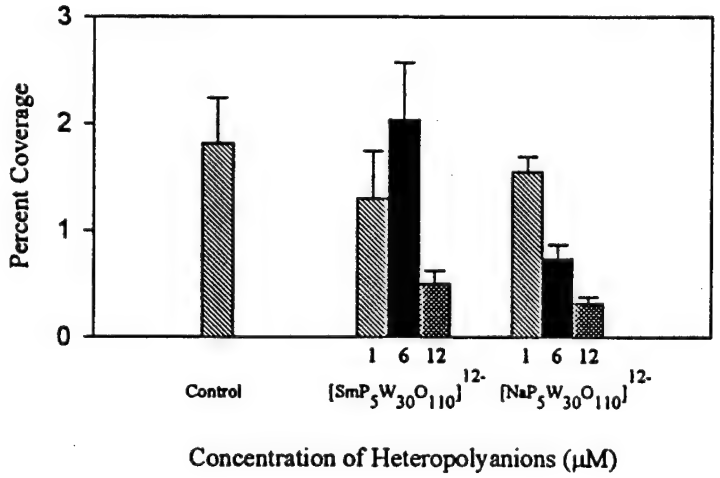
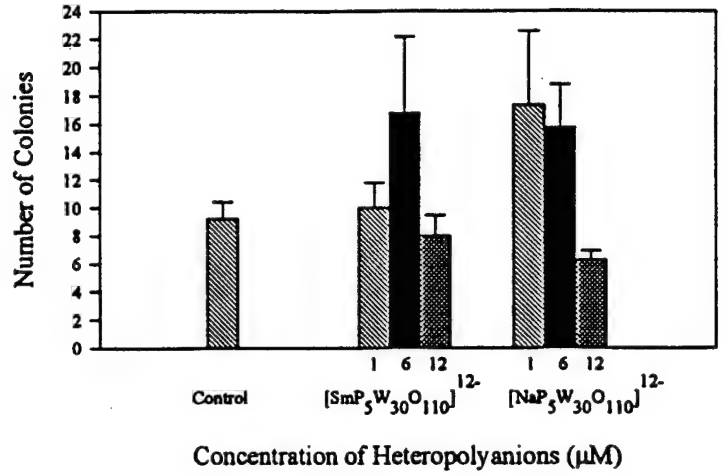
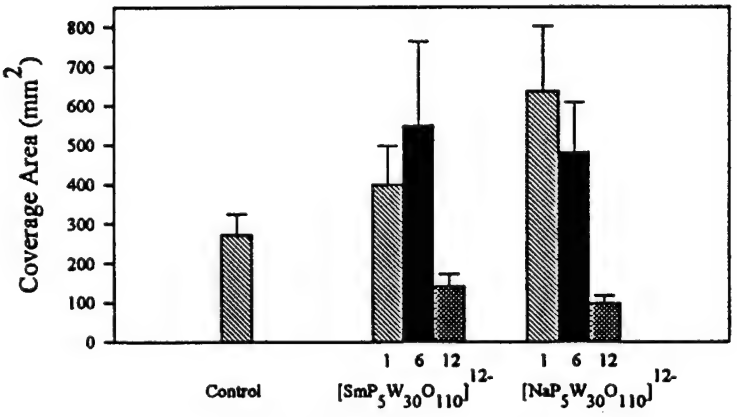


Figure 4. 2 weeks pulse





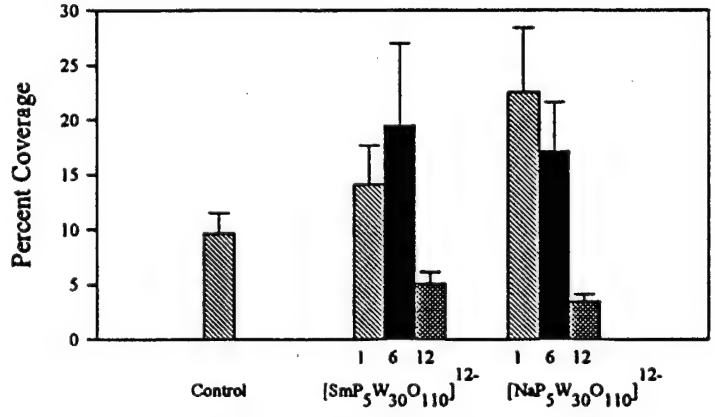
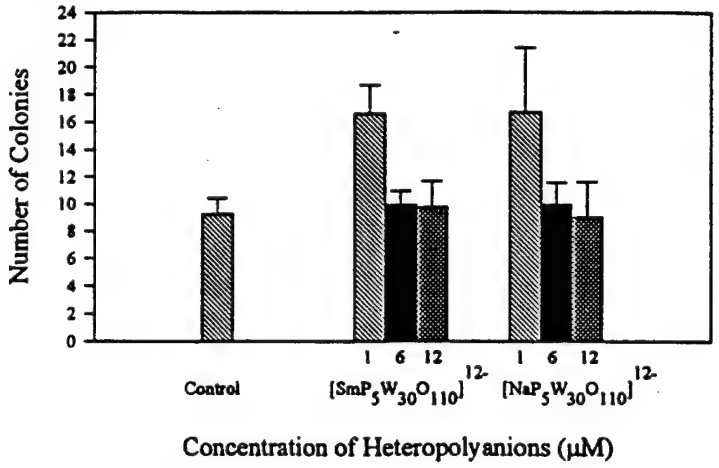
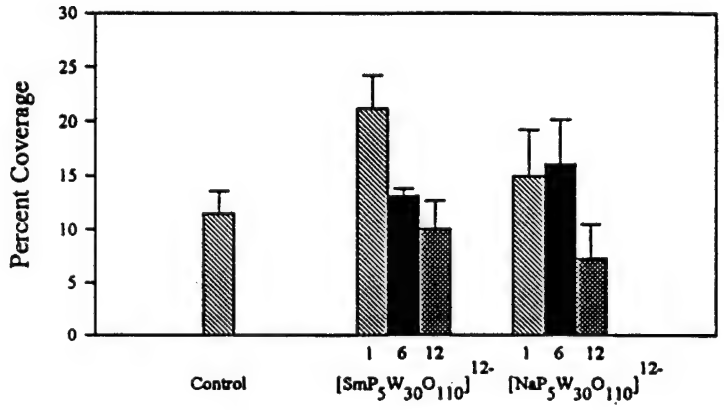


Figure 5. 4 weeks continuous





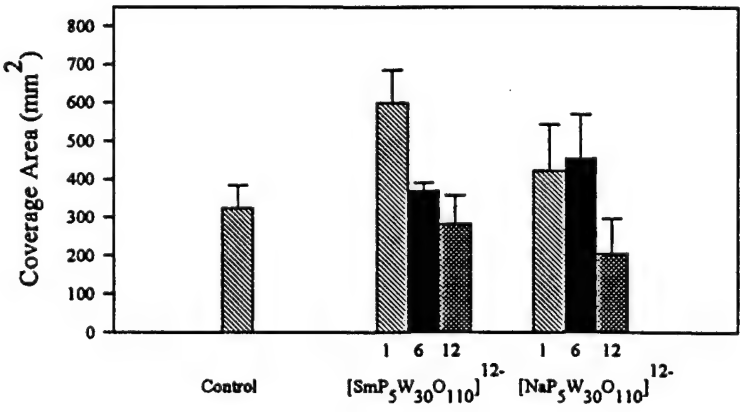
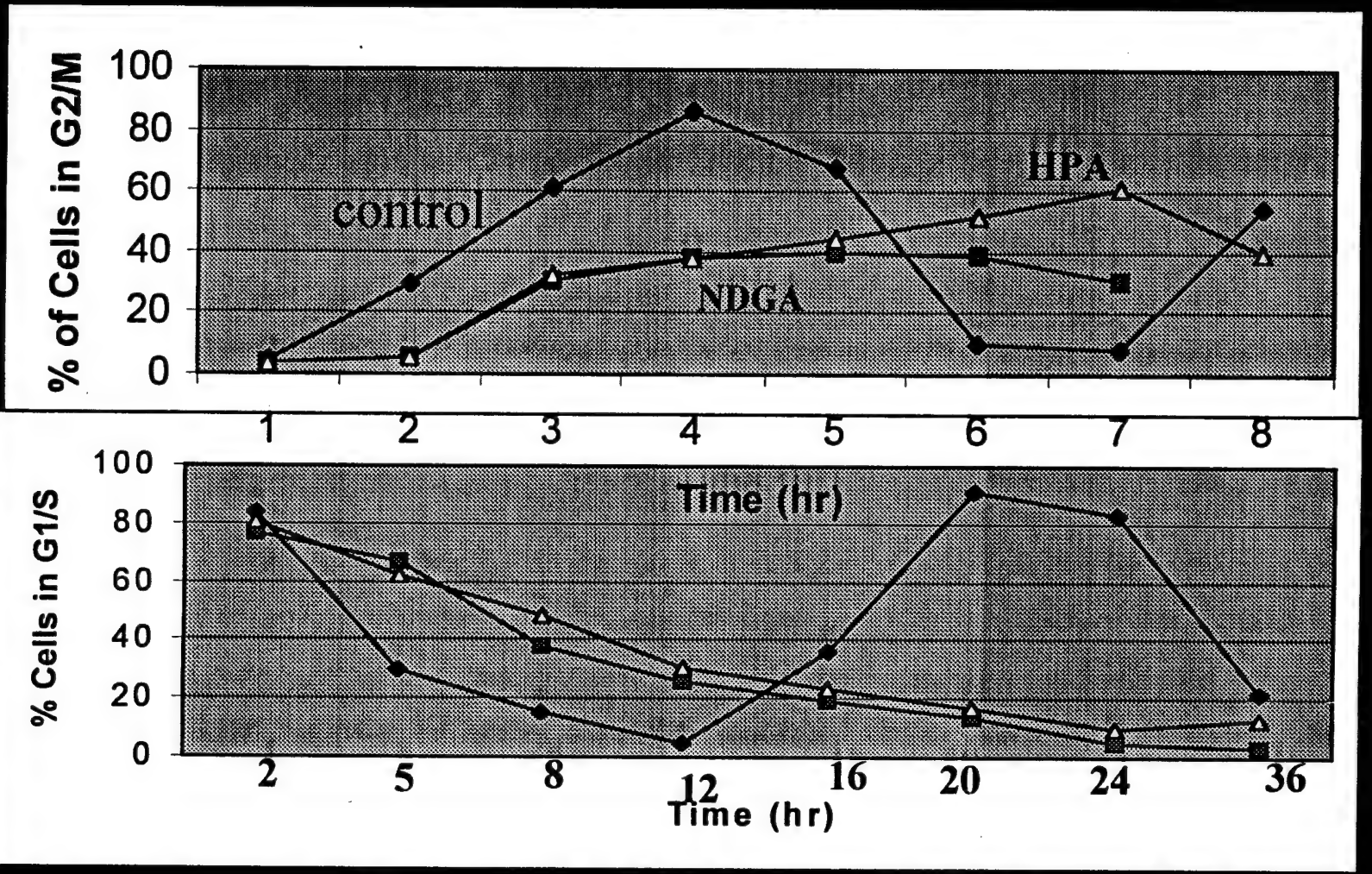


Figure 6. 4 weeks pulse


% of Cells in G2/M
100
80
60
40
20
0
control
HPA
NDGA
1
2
3
4
5
6
7
8
% Cells in G1/S
100
80
60
40
20
0
Time (hr)
2
5
8
12
16
20
24
36
Time (hr)

Fatty acid binding protein involvement in utilization/trafficking of mitogenic lipids

Intracellular utilization of bioactive lipids is a critical component in the process by which these molecules continuously stimulate proliferation through nuclear receptor interactions. Transport/ utilization are proposed to be mediated by the cytoplasmic proteins known as fatty acid binding proteins (FABPs) [1]. These proteins may also be important in regulating intracellular fatty acid concentrations. Such crucial participation in the trafficking and availability of fatty acids implicate FABPs as critical links in the mechanistic chain connecting dietary fat with cancer.

FABPs are found in abundance in a variety of tissues. The members of this broad multigene family currently consist of at least seven types whose amino acid sequences were determined from purified proteins or cDNA nucleotide sequences from tissue RNA [2-8]. Initially, an FABP was named for the tissue from which it was isolated and includes: 1) adipocyte (A-FABP), 2) heart or muscle (H-FABP), 3) brain (B-FABP), 4) epidermis or psoriasis-associated (E-FABP), 5) liver (L-FABP), 6) intestine (I-FABP), and 7) myelin or P2 (P2-FABP). A frequently studied FABP from bovine mammary gland, designated MDGI (mammary-derived growth inhibitor), thought to be a distinct type in itself, was later identified as H-FABP [9, 10]. Expression of each FABP type is not necessarily limited to the tissue from which it was originally isolated. In some tissues FABP expression is developmentally regulated and different types may be expressed in different regions of an organ.

The properties common to FABPs include their intracellular abundance, their small size (a molecular weight range of 14-16 kDa and an average of 132 amino acids), their sequence relatedness and three-dimensional structure and their ability to bind a variety of lipids. As a group A-FABP, H-FABP, B-FABP, and E-FABP in humans share between 50-65% protein sequence homology and contain a tyrosine near residue 20 that can be phosphorylated. These four FABPs share only 20-25% homology with L-FABP or I-FABP which do not have the tyrosine. L-FABP is distinguished by its lack of the amino acid tryptophan.

Certain FABPs have been reported to have differential effects on cell growth when cDNA clones have been transfected into cells. Transfection of L-FABP into hepatoma cells increased proliferation [1, 11, 12]. In contrast, MDGI (H-FABP) appears only in normal and not tumor mammary cells [13-14]; transfection of a cDNA clone of MDGI into breast cancer cells or mouse mammary epithelial cells resulted in loss of tumorigenicity [15]. FABPs are known to bind many different groups of fatty acids and their derivatives, and other bioactive lipids [reviewed in [16]. L-FABP exhibits different lipid binding characteristics from that of A-FABP or H-FABP. L-FABP transfected into rat hepatoma cells also mediates cell induction by carcinogenic peroxisome proliferators [17], and involves alteration of eicosanoid metabolism (Panandiker et al [addendum J-B]). FABP causes a net diffusion of fatty acids from to intracellular membrane compartments [18, 19].

Changes in expression of FABPs have been reported for bladder cancer. Psoriasis-associated FABP (E-FABP) was noted to increase in level with increase in differentiation of bladder squamous cell carcinomas [20]. Although FABPs are intracellular proteins, H-FABP has been detected in elevated levels in plasma and urine of patients suffering from myocardial infarction [21-22], whereas psoriasis-associated FABP (E-FABP) was among a number of marker proteins detected in the urine of bladder cancer patients [23]. In addition, loss of adipocyte-FABP (A-FABP) was reported with progression of human bladder transitional cell carcinomas [24]. The presence of A-FABP correlated with the grade and stage of the disease. The A-FABP protein was present in high levels in grade I and II TCCs whereas grade III had 37% reduction and grade IV had no A-FABP expression. A-FABP may act as a growth inhibitor similar to the MDGI (H-FABP) protein in breast cancer and loss of A-FABP expression may serve as a prognostic marker for aggressive bladder cancer. Our preliminary data are the only studies of the different levels of FABPs in normal and cancerous breast cells.

MDGI has been regarded as being associated with the "normal" breast since it is singularly absent in the breast cancer cells/tissues examined and when added exogenously, blocks growth of breast cancer cells in vitro. However, little note has been taken of the fact that it is absent in "normal" breast tissue, but is abundantly produced during lactation (specialized normal). We suggest that the role of MDGI, in that specialized state, is to bind, and thus, protect the epithelial cells from the incredibly rich mileu of bioactive lipids and other factors that bath the cells during lactation. We postulated that other FABPs were playing a role in normal/tumor breast cells; we now have preliminary data implicating an altered balance of FABPs in normal vs tumor breast cells.

Current studies in our laboratory regarding fatty acid binding proteins in breast cancer: We have found, using RT-PCR primer pairs based on the cDNA for L- and I-FABPs (Figures 1b, a), 14- and 3-fold increases in tumor vs normal breast cells. In contrast, RT-PCR for A-FABP (Fig 1c) showed a 7-fold decrease in the expression level in cancer vs normal cells. A-FABP, heart-FABP and MDGI are similar in sequence and are found associated with normal cells. Differences were seen between T-47D and MCF-7 cells in response to L-FABP (Fig 1b). These results suggests that A-FABP may act as a tumor suppressors in breast cells similar to MDGI (H-FABP) in MCF-7 cells [15]. We are currently examining L- and I-FABPs and cell cycle patterns [addendum J-Fig. D] in MDGI sham and sense transfected MCF-7 cells and would predict that MDGI sense-containing cells show suppression of I-FABP . Of the few FABPs tested so far, individually, each of these have been shown, in other cell systems, to correlate with the normal or tumor state. Our study is the first report to show decreases in the heart-type FABPs (A-FABP) **concomitant** with increases in mitosis promoting FABPs (I-FABP and L-FABP). We have synthesized a series of drugs, heteropolyanions (HPA), which are free-radical scavengers and target eicosanoid metabolism [1]. Use of HPA-Na, blocked cell cycle progression of MCF-7 cells and altered relative ratios of A- and I-FABP in cultures of tumor cells [addendum B-Figures A-D & F].

Expression of Various Fatty Acid Binding Proteins in Breast Cells.

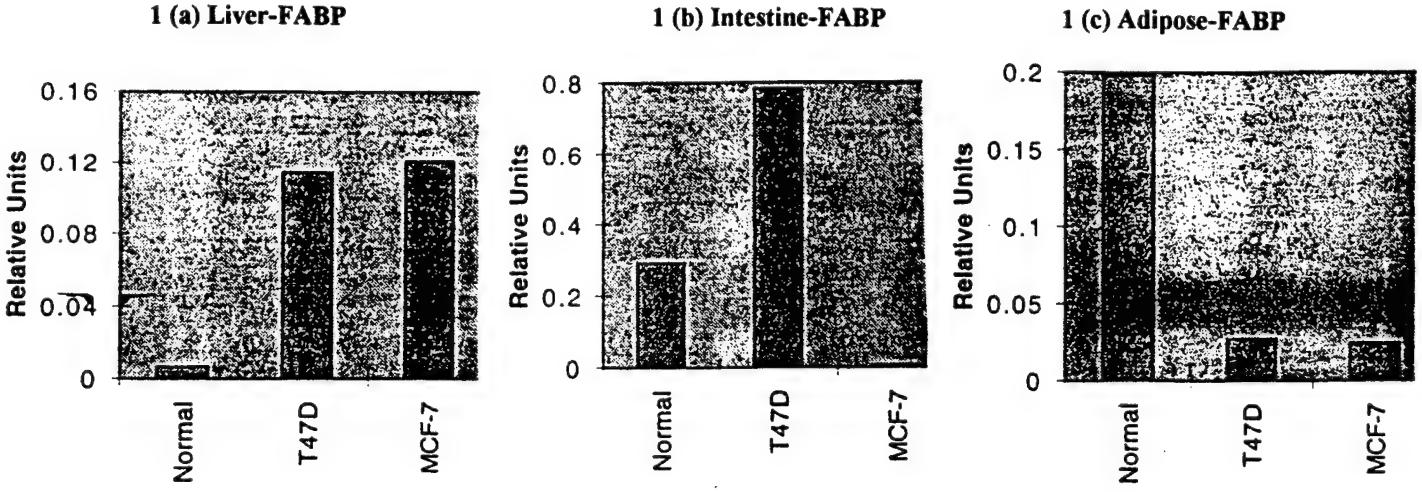


Figure 1. RNA was extracted from breast normal and tumor cultured cells. PCR primers for Liver-(a), Intestine-(b) and Adipose-FABPs (c) were used to determine the levels of expression of the specific FABP.

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Target Protein of Four Classes of Genotoxic and Nongenotoxic Liver Carcinogens Acts with a Potent Peroxisome Proliferator to Depress Specific Metabolites of Arachidonic Acid in Their Induction of Mitogenesis

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Running Title: Specific Eicosanoids during Mitogenesis

FOOTNOTES

[1] This work was supported in part by National Institutes of Health Grant CA05945, institutional grant CA06927 from the National Institutes of Health, and an appropriation from the Commonwealth of Pennsylvania. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute, or the Department of the Army, or the Department of Defense (Para 4-3) AR360-5.

[2] This manuscript is dedicated to the memory of Sam Sorof, Ph.D. (1926-1996).

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[4] The abbreviations used are: L-FABP, liver fatty acid binding protein; H-FABP, heart fatty acid binding protein; MDGI, mammary-derived growth inhibitor; Wy-14,643, 4-chloro-6-(2,3-xylidino)-2-pyrimidylthioacetic acid; PBS, phosphate-buffered saline; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTB_4 oxid, leukotriene B_4 oxidized; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; TXB_2, thromboxane B_2; PGE_2, prostaglandin E_2; $6KPGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; LXA_4, lipoxin A_4; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-HETE-Me, 5-hydroxyeicosatetraenoic acid methyl ester; 5-HETE-δ-Lac, 5-hydroxyeicosatetraenoic acid-δ-lactone; 12-HETE, 12-hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 15-HETE-Me, 15-hydroxyeicosatetraenoic acid methyl ester.

ABSTRACT

Two classes of nongenotoxic liver carcinogens, and two groups of genotoxic liver carcinogens were shown previously to bind specifically to rat liver fatty acid binding protein (L-FABP[3]) *in vitro* and/or *in vivo*. In addition, the induction of mitogenesis by the two classes of nongenotoxic hepatocarcinogenic peroxisome proliferators depended on the expression of transfected L-FABP, and correlated directly with the affinities of the protein for the peroxisome proliferators *in vitro*. Inasmuch as L-FABP binds many cell regulatory metabolites of arachidonic acid (eicosanoids) *in vitro*, the present study examined the differential effects in the kinds and levels of 20 intracellular and extracellular eicosanoids for significant differences that are attributable to the expression *versus* the nonexpression of transfected L-FABP, and also to the presence of the potent nongenotoxic hepatocarcinogenic peroxisome proliferator, Wy-14,643, during mitogenic induction by both agents. Employed was the novel use of the differential cell system, in which Wy-14,643 previously induced maximal mitogenesis in sense transfected L-FABP expressing cells, but not in the antisense transfected L-FABP nonexpressing cells, both transfectants derived from the same rat hepatoma cell line that expressed neither L-FABP nor its mRNA. The transfected L-FABP expressing and nonexpressing cell lines were incubated with [^{3}H]arachidonic acid for seven days in the presence or absence of Wy-14,643 (10^{-7} M) without change of culture media. The differential comparative analysis eliminated as insignificant all effects that were common in sense *versus* antisense transfected cell types and analytical treatments. All procedures were stringently controlled for maximal resolution and reproducibility, and minimal chemical instability. Twenty eicosanoids of the cells and culture media were separated chromatographically and quantified, 18 were identified by use of authentic reference standards, and the identities and levels of 8 were confirmed by quantitative immunofluorescence. Many intracellular and extracellular eicosanoids were significantly depressed in amount by L-FABP and Wy-14,643, when the effects of both agents were considered together. In contrast, by uses of three control protocols that were previously found not to induce mitogenesis because of the lack of either or both L-FABP and Wy-14,643 analyses of significant differences revealed that L-FABP and Wy-14,643 each acted individually with great specificity during the induction of the mitogenesis by both agents. Thus, L-FABP depressed the levels of only a triad of metabolites derived from leukotriene A_4, namely intracellular leukotrienes C_4 and B_4 and extracellular lipoxin A_4, suggesting that the primary overall depression by L-FABP was of intracellular leukotriene A_4. Likewise, Wy-14,643 depressed the intracellular levels of only a triad of known cell regulatory HETE/HPETEs, namely 12-hydroxyeicosatetraenoic acid, 5-hydroperoxyeicosatetraenoic acid, and apparently transiently 5-hydroxyeicosatetraenoic acid. All other effects, including those due to secondary biotransformations and chemical instabilities, apparently did not produce significant differences. Evident also were significant depressions of eicosanoids due to interactive effects between the two triads and possibly with other cellular constituents. Thus, L-FABP and WY-14,643 each depressed a different triad of cell regulatory eicosanoids during the induction of mitogenesis. The present findings provide the basis for identification of the mediators by which the expression of a protein target, namely L-FABP, of four classes of genotoxic and nongenotoxic liver carcinogens and a potent nongenotoxic, hepatocarcinogenic peroxisome proliferator induce hepatocyte multiplication while causing depressions of specific eicosanoids.

INTRODUCTION

Four classes of nongenotoxic and genotoxic liver carcinogens bind specifically to rat liver fatty acid binding protein (L-FABP) *in vitro* and/or *in vivo* (1-7). Previous evidence indicates that this abundant 14 kDa cytoplasmic protein is not only an intracellular carrier of fatty acids, but is also an inducer and a modulator of cell proliferation (mitogenesis) in rat hepatocytes (reviewed 8), a process thought to be essential in oncogenesis (9,10). Thus, the inductions of mitogenesis by two classes of hepatocarcinogenic nongenotoxic peroxisome proliferators (amphipathic carboxylates and tetrazole-substituted acetophenones) in L-FABP transfected cells not only depend on the expression of L-FABP (11), but also the magnitude of the induction of mitogenesis correlates with the relative affinities of the peroxisome proliferator for the protein *in vitro* (7,11). In addition, L-FABP is a specific protein target of reactive metabolites of the genotoxic carcinogens, 2-acetylaminofluorene and aminoazo dyes, during liver carcinogenesis in rats (1-6), and the accumulations of the resultant adducts agree temporally with the accompanying growth inhibition of hepatocytes (2-4). Further, together with its unsaturated fatty acid ligands, L-FABP promotes DNA synthesis and cell division, maintenance of cell morphology, and cell survival of L-FABP transfected rat hepatoma cells (12,13). Moreover, L-FABP binds many cell regulatory metabolites of arachidonic acid (eicosanoids) *in vitro* (8,14,15), among which eight prostaglandins exhibit relative avidities for L-FABP that correlate with their growth inhibitory activities toward cultured hepatocytes (16). Lastly, the level of L-FABP is markedly elevated specifically during mitosis of hepatocytes in normal and regenerating rat livers (17-19), but in contrast is increased throughout the cell cycle in hyperplastic and malignant hepatocytes during carcinogenesis by

2-acetylaminofluorene and aminoazo dyes (17, 20). The principle has, therefore, emerged that the cell regulatory activities expressed by L-FABP are dependent on the noncovalent binding of its normal ligands, *i.e.*, unsaturated fatty acids and eicosanoids, and its competitive noncovalent binders, *e.g.*, peroxisome proliferators, and to its covalent reactions that arise from the genotoxic hepatocarcinogens, *e.g.*, 2-acetylaminofluorene and aminoazo dyes.

FABPs constitute a family of proteins. In addition to L-FABP, several members of the family are associated with modulation of cell proliferation (reviewed 8). Notably, the potent mammary-derived growth inhibitor (MDGI) and heart (H)-FABP are coexistent in bovine lactating mammary glands (21), and are 95%-homologous in peptide sequence (22). Both proteins inhibit DNA synthesis, and MDGI reversibly suppresses the multiplication of a variety of cultured epithelial cells. In accord, the growth inhibitory activity of recombinant bovine MDGI mutually opposes the growth stimulatory activity of EGF in whole organ cultures of mouse mammary glands (21, 22). Lastly, the gene of bovine MDGI is a potent tumor suppresser of human breast cancer cell lines that expresses mitogenic inhibitory activities comparable in magnitude to those of the tumor suppressers *Rb*, *p53* and *H19* (23).

In order to gain insight into the mechanisms of the many activities of L-FABP, a cultured rat hepatoma cell line that does not express L-FABP and its mRNA was stably transfected with rat L-FABP cDNA (12). Cells transfected with the L-FABP sense cDNA expressed the recombinant L-FABP mRNA and protein, while those transfected with the antisense cDNA did neither. Significant differences in constituents and activities between the two types of tranfected cell lines have been attributed to the expression of L-FABP. The system constitutes a homologous, zero-background, cell model suitable for internally controlled and differential studies of the many roles of L-FABP in rat hepatocytes.

Metabolites of arachidonic acid exhibit multiple cell regulatory activities including modulation of cell growth (cited in 8). Inasmuch as a variety of these cell regulatory eicosanoids bind avidly to L-FABP *in vitro* (14,15), the present study asked whether there are specific differences between the levels and kinds of eicosanoids of the L-FABP expressing cells *versus* those of the L-FABP nonexpressing cells during the induction of mitogenesis by both L-FABP and the potent hepatocarcinogenic peroxisome proliferator, Wy-14,643, a nongenotoxic amphipathic carboxylate. We report here that L-FABP and Wy-14,643 each act with great specificity, each depressing the levels of only a triad of cell regulatory biochemically related eicosanoids during the induction of mitogenesis. The present findings provide the basis for the identification of the mediators by which the expression of a target protein (L-FABP) of four classes of liver carcinogens, acting together with a potent hepatocarcinogenic peroxisome proliferator, induce hepatocyte multiplication.

MATERIALS AND METHODS

Cells. Rat hepatoma HTC-R_3T_2 cells that did not express detectable L-FABP mRNA or protein were previously transfected with sense and antisense L-FABP cDNAs (12). Stable expression of the transfected cDNA was under the promoter control of the long terminal repeat of Rous sarcoma virus. Cloned transfected cell lines were routinely passaged in RPMI 1640 medium supplemented with 10% calf serum (both from GIBCO) (11-13). However, in experiments the cells were grown in that medium containing 1% calf serum (12).

Transfected Hepatoma Cells for Differential Analyses. Cells were cultured using the same conditions that previously produced maximal L-FABP-dependent induction of mitogenesis in synergy with the potent peroxisome proliferator Wy-14,643 (10^{-7} M) in 1% calf serum (11). Sense-transfected S7 and antisense-transfected A1 hepatoma cells (2.8×10^5), passaged from log phase cultures in RPMI 1640 (without indicator) and 10% calf serum, were plated in duplicate in 20 ml of that medium in 150 mm diameter tissue culture dishes (Nunclon Delta, Nunc). After 18 hrs at 37°C in a humidified atmosphere of 5% CO_2, the media were replaced with 20 ml of RPMI 1640 (no indicator) containing 1% calf serum, Wy-14,643 (Chemsyn Science Laboratories, Lenexa, KA; freshly dissolved in ethanol) at 10^{-7} M (final), and also 1.25 µCi/ml of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (200 Ci/mmole, 1 mCi/ml ethanol; American Radiolabeled Chemicals Inc., St. Louis, MO). The final concentration of the labeled arachidonic acid in the media was 6 nM in 0.225% ethanol. Control cultures of each cell type were given the same medium without Wy-14,643. The media were not renewed over the seven-day period of cell incubations for necessary and beneficial reasons. See Discussion. Beginning with the preparation of the [^{3}H]arachidonic acid solution, all operations were carried out under dim light. At the end of the incubation period, the media and two washings with PBS (each 5 ml) were pooled. The cells in each dish were immediately scraped and washed three times with 3 ml of PBS. The cell suspensions with washings (*ca.* 9 ml) and the media with washings (*ca.* 29 ml) were immediately frozen in dry ice, and sent overnight to the collaborating laboratory as blind coded samples.

Analyses of Eicosanoids. The cell suspensions and media were thawed within 24 hrs of receipt. [^{14}C]-eicosatrienoic acid (20,000 dpm; American Radiolabeled Chemicals, St. Louis, MO) and nonradioactive 15(S)-hydroxyeicosa-11Z,13E-

dienoic acid (5 ng) were then added to each sample as internal standards for corrections of recoveries of all metabolites and as markers in HPLC elution gradients, respectively. After immediate centrifugation of the cells at 3,000 x g, the supernatant fluids were kept in 50 ml polypropylene tubes at 0°C, while the pellets were resuspended in 500 µl of aqueous methanol (1:1), sonicated with three bursts on ice, and recentrifuged. Both supernatant fluids were combined, diluted (4x) with acidified distilled water to pH 4, and recentrifuged to remove any precipitate. Meanwhile, the defrosted culture media were immediately diluted (4x) with acidified distilled water to pH 4, and clarified by centrifugation as above. During processing all samples were kept on ice or refrigerated; otherwise they were stored at -84°C under nitrogen. Arachidonic acid metabolites were removed from aqueous solutions by use of C18 disposable cartridges (Sep pak, Waters, Milford, MA; activated with 4 ml of methanol, washed with 10 ml of distilled water, and submerged in distilled water up to a few hours). One end of the cartridge was attached to a loaded syringe; the other end was inserted into a rubber stopper mounted in the wall of a vacuum manifold (Millipore, Milford, MA). Vacuum was adjusted so that the aqueous solution flowed (*ca.* 5 ml/min) from the loaded syringe to the manifold's waste trap. After the sample was completely applied to the cartridge, the feeder syringe and cartridge were washed with 4 ml of 2% ethanol. Metabolites still bound to the cartridge were eluted immediately using 10 ml of acetonitrile:methanol (85:15) and collected in a 15 ml polypropylene tube in ice. The samples were then stored at -84°C under nitrogen (at day 3 since the harvests of the cells and media). After drying in a concentrator-evaporator (Savant Instruments, Inc., Farmingdale, NY), each sample was collected at the bottom of a 15 ml polypropylene tube with five 250 µl volumes of methanol, dried again, and dissolved in 200 µl of methanol. After exhaustive vortexing and centrifugation, the supernatant fluid was transferred to an HPLC injection vial using a 300 µl insert. The tube and pellet were washed with 100 µl of methanol, centrifuged, and the resultant and previous supernatant fluids were combined (total *ca.* 270 µl). Volumes of 240 µl were resolved by HPLC employing essentially the procedure of Boyle *et al.* (24). Eicosanoids were identified on the basis of the coincidences of their internally corrected elution positions with those of authentic reference standards (BioMol, Plymouth Meeting, PA). An in-line radioactivity detector (Packard Instruments, Downers Grove, IL) was set at 6 sec intervals for integration updates based essentially on least squares analysis of the data. Peaks were detected automatically by changes in slopes relative to time. The recovery of the [^{14}C]-internal standard provided the correction factor used in quantifications of all eluted metabolites. The contents of the eicosanoids in the cell suspensions and media were then normalized on the bases of both the DNA in their respective cell suspensions and the volumes of their original pools (*ca.* 9 ml and 30 ml, respectively). Statistical significance was determined by the two-tailed Mann-Whitney U test.

Quantitative Immunofluorescence Assays. The validity of the identifications and quantifications of the chromatographically separated radioactive eicosanoids was tested by quantitative immunofluorescence or elisa assays (Perseptive Diagnostics, Boston, MA). The metabolites of eight chromatographic peaks from an extract of L-FABP expressing cells not treated with Wy-14,643 were analyzed in quintuplicate (Table 1). 5-HETE was employed as the internal reference compound for comparisons of the relative radioactivity of the HPLC separated peaks and the immunofluorescence data.

RESULTS

Intracellular and Extracellular Eicosanoids. The stably transfected L-FABP expressing S7 and nonexpressing A1 hepatoma cells were subjected to the procedure that previously induced maximal L-FABP-dependent mitogenesis by Wy-14,643 in 1% calf serum over seven days and did not induce mitogenesis in the absence of either agent (11). The Wy-14,643, which was maximally mitogenic at 10^{-7} M, was omitted in control experiments. Twenty eicosanoids were isolated from the cells and media in six experiments of each protocol. All eicosanoids were quantified, and 18 were identified (Fig. 1). It is noteworthy that the immediate derivatives of the labeled arachidonic acid were generated by the cells, rather than by the serum or chemical instability, insofar as without cells, incubations of the complete medium for seven days without fluid change failed to yield any chromatographically resolved derivative (with or without Wy-14,643 at 10^{-7} M).

Quantitative Immunofluorescence. The identities and amounts of eight of the eicosanoids that co-eluted chromatographically with authentic reference standards, as shown in Fig. 1, were confirmed by assays of quantitative immunofluorescence. Comparisons of the ratios of these metabolites relative to 5-HETE showed that there was no significant difference in the results obtained by the two methods of analysis (Table 1).

Protocols to Resolve the Individual Actions of L-FABP and Wy-14,643. Based on our previous findings (11), three types of mitogenically non-induced cells were employed as controls (L-FABP expressing cells without the peroxisome proliferator; and L-FABP nonexpressing cells with and without the proliferator). By comparing these three individual types of mitogenically non-induced cells *versus* the mitogenically induced cells (L-FABP expression with the proliferator) the alterations of the eicosanoid levels that were brought about jointly by both the L-FABP expression and Wy-14,643 were

dissected into the differential changes that were due to each agent individually. The remaining differences were attributed to interactive effects between the two agonists themselves, and possibly also with other cellular constituents.

Multiple Depressions by Joint Actions of L-FABP and Wy-14,643. Because the induction of mitogenesis depended on both L-FABP expression and the actions of Wy-14,643 (11), the combined effects of their dual presence *versus* their absence on the levels of eicosanoids were first compared in the cells and media. Of the 20 intracellular eicosanoids shown in Fig. 1, 16 were significantly reduced in amount. In the culture media, the levels of four (LXA_4, 12-HETE, 15-HETE-Me, and Unknown I) of the 20 eicosanoids were significantly depressed, but at higher than their intracellular levels, suggestive of active passage out of the cells.

Specific Effects of L-FABP Expression: Depressions of the Triad-LTC_4, LTB_4 and LXA_4. The above multiplicity of the eicosanoid depressions by the joint actions of L-FABP expression and the peroxisome proliferator led us to analyze the effects of L-FABP expression itself under the conditions that were shown previously to induce mitogenesis. Comparisons of the levels of the eicosanoids of the L-FABP expressing cells *versus* those of the L-FABP nonexpressing cells revealed that the levels of only three of the 20 intracellular eicosanoids were significantly different following the seven days of exposure to Wy-14,643. The intracellular levels of LTB_4, LTC_4, and LTD_4 were significantly lower in the L-FABP expressing cells than in the L-FABP nonexpressing cells (Fig. 2, upper). However, unlike with LTB_4 and LTC_4, the depression of LTD_4 was not associated with the induction of mitogenesis (see below). Only the level of LXA_4 was significantly lower in the culture media of the L-FABP expressing cells exposed to Wy-14,643 (Fig. 2, upper). Thus, during the induction of mitogenesis (11), L-FABP expression itself acted specifically to depress the levels of only the two intracellular leukotrienes B_4 and C_4 and extracellular lipoxin A_4.

In the absence of Wy-14,643 [no induced mitogenesis(11)], L-FABP expression brought about decreases in the levels of eicosanoids that were different from those above, with one exception. Comparisons of the L-FABP expressing *versus* nonexpressing cells revealed that the expressing cells had significantly lower levels of five intracellular eicosanoids, namely LTD_4, LTE_4, Unknown 2, 15-HETE, and 5-HETE (Fig. 2, lower). Meanwhile, the media contained only significantly lower amounts of the two prostaglandins, PGE_2 and $PGF_{2\alpha}$ (Fig. 2, lower). Thus, without the induced mitogenesis due to the absence of Wy-14,643, L-FABP expression was accompanied by decreases of five intracellular and two extracellular eicosanoids. All of these eicosanoid changes differed from those that occurred during the induction of mitogenesis by the peroxisome proliferator in the L-FABP expressing cells, with the one exception of that of LTD_4. The LTD_4 was depressed in both the presence and absence of the peroxisome proliferator (Fig. 2, upper and lower). The net result is that only the decreases in the levels of the eicosanoid triad, namely, intracellular leukotrienes LTC_4 and LTB_4 and extracellular lipoxin LXA_4, were associated with the expression of L-FABP expression during the induction of mitogenesis.

Specific Effects of Peroxisome Proliferator: Intracellular Depressions of the Triad-12-HETE, 5-HETE and 5-HPETE in Cells. The results of exposure *versus* nonexposure to Wy-14,643 were compared in the cultures of the L-FABP expressing and nonexpressing cells (Fig. 3). Of the 20 quantified intracellular eicosanoids, only four were affected by Wy-14,643 during the mitogenesis induced in L-FABP expressing cells. Only 5-HPETE, 5-HETE-Me, 5-HETE-δ-Lac, and 12-HETE were significantly lowered intracellularly in the L-FABP expressing cells by the treatment with Wy-14,643 (Fig. 3, upper). The methyl ester and the lactone originate metabolically mainly from 5-HETE, which itself derives from 5-HPETE. In contrast, no extracellular eicosanoid was significantly lowered by Wy-14,643 (Fig. 3, upper). In summary, exposure of the L-FABP expressing cells to Wy-14,643 resulted in decreased intracellular levels of the triad, 12-HETE, 5-HPETE, and 5-HETE apparently transitionally, based on comparisons with the nonexpressing cells.

In control experiments with L-FABP nonexpressing cells [no induced mitogenesis (11)], Wy-14,643 significantly decreased the intracellular levels of LTD_4, LTE_4, and 5-HETE, and extracellular 5-HPETE (Fig. 3, lower). Thus, the expression of L-FABP determined which eicosanoids were decreased by Wy-14,643.

Interactive Effects between L-FABP and Wy-14,643 and Other Cellular Constituents. The above individual effects of L-FABP and Wy-14,643 together accounted for a minor number of the significant depressions of the eicosanoids presented in Fig. 1. The remaining depressions were attributed to unknown interactive effects between L-FABP expression and Wy-14,643 themselves, and also possibly with other cellular constituents.

DISCUSSION

Expression of L-FABP, which is the target protein of four classes of genotoxic and nongenotoxic hepatocarcingens and its ligand, the potent liver carcinogenic peroxisome proliferator, Wy-14,643, together previously induced mitogenesis in sense-transfected L-FABP expressing hepatoma cells, and not in antisense-transfected L-FABP nonexpressing hepatoma cells (11). The induction of mitogenesis has now been found to be associated with highly specific depressions of eicosanoids. L-FABP expression itself lowered the levels of only the triad of LTA_4-derived metabolites, LTC_4, LTB_4 and LXA_4, during the induction of

mitogenesis (Fig. 2), suggesting that the core depression was that of their intracellular leukotriene precursor, LTA_4. Likewise, Wy-14,643 itself decreased the intracellular levels of 5-HPETE, 5-HETE-Me, 5-HETE-δ-Lac, and 12-HETE. Of these, 5-HPETE and 12-HETE during the induction of mitogenesis (Fig. 3). Of these, 5-HPETE and 12-HETE are main line metabolites in the arachidonic acid pathway, while 5-HETE-Me and 5-HETE-δ-Lac are branch metabolites whose common lowerings likely stemmed from that of 5-HETE. Although the formation of the methyl ester and lactone metabolites can not be ruled out as sites of these depressions, its likelihood seems outweighed by the concurrent decreases of 5-HPETE and 12-HETE, which together with the 5-HETE metabolites form a triad of related intracellular HPETE/HETEs whose lowerings may reflect perturbations at the level of the initial generation of arachidonate derivatives. It is notable that in another study the extracellular levels of 5-HETE-Me, 5-HETE-δ-Lac and 15-HETE-Me were also lowered by L-FABP expression after incubation of the transfected cells in serum-free medium for 24 hrs (A.W. Panandiker, T. Boyle, M. Jett, and S. Sorof, unpublished results). Hence, the levels of eicosanoids of the three series of 5-, 12-, and 15-HPETE/HETEs were lowered by L-FABP expression or actions of Wy-14,643 in two protocols, adding to the likelihood that the core perturbations were connected with the HPETE/HETEs. Accordingly, in the present study, Wy-14,643 itself decreased the intracellular levels of only the triad, 5-HPETE, apparently 5-HETE transitionally, and 12-HETE, during the induced mitogenesis. This combination of depressions occurred in the three control cultures that were previously shown not to induce mitogenesis (11). All of these decreases by the two agents were intracellular, except for that of LXA_4, whose lowering was also extracellular, likely reflecting a decrease of its intracellular leukotriene precursor, LTA_4. As with most activities of L-FABP, the role of Wy-14,643 in the induction of mitogenesis may involve the previously reported noncovalent binding of the peroxisome proliferator to L-FABP (7, reviewed 8). It therefore seems logical to speculate that that binding interaction between L-FABP and Wy-14,643 may bring about the specific intracellular depressions of the two triads of cell regulatory and biochemically related eicosanoids as part of their induction of mitogenesis.

The depressions of eicosanoids by L-FABP expression and Wy-14,643 may result from at least three factors. Firstly, as the conventionally accepted intracellular carrier of fatty acids including arachidonic acid, L-FABP may facilitate their utilization for energy and membrane generation. Secondly, L-FABP binds avidly 5-, 12-, and 15-HPETEs and their HETE metabolites *in vitro* (15). As ligands bound to L-FABP, these metabolites may be less available for biosynthesis of downstream eicosanoids. Thirdly, Wy-14,643 and other amphipathic peroxisome proliferators behave as poorly metabolizable analogs of fatty acids. They mimic fatty acids in regard to enzyme inductions in mitochondria (25-26) and peroxisomes (27), peroxisome proliferation by long chain fatty acids and fatty acid analogs (26-28), formation of acyl-CoA esters (29), elevation of P450IVA1 ω-hydroxylase (25, 29), activation of mouse peroxisome proliferator-activated receptor (30), binding to L-FABP (7,12,13), and synergy with L-FABP in induction of mitogenesis (11-13). It seems reasonable therefore to suggest that acting as a competitive analog of fatty acids Wy-14,643 may inhibit the metabolic conversion of arachidonic acid to eicosanoids.

The protocol of the present study contained several features that were both important and beneficial. Firstly, it employed the powerful procedure of differential comparative analysis to detect significant differences in the levels of eicosanoids of the sense-transfected L-FABP expressing cells *versus* those of the antisense-transfected L-FABP nonexpressing cells and also cells that were treated *versus* those not treated with WY-14,643 (11). Eliminated as insignificant were all changes, including those due to biotransformations and chemical instabilities, that were similar in the two types of cells and treatments. Where there were significant differences in the levels of eicosanoids, their relative relationships are presumed to be retained during biotransformations and chemical instabilities. In those cases, the demonstrated specificities of the significant differences enabled deductions to be made concerning the courses of the secondary alterations of the eicosanoids. Secondly, the protocol was the same as that of the previous investigation on the induction of cell proliferation by L-FABP and Wy-14,643 (11). As in the former study, the culture media were not changed during the seven days of cell incubations with the labeled arachidonic acid. It is important to stress that the cells required three days of exposure to L-FABP and Wy-14,643 before the start of mitogenesis (11). Renewal of the media would have impaired the conditioning of the cultures, altered the dynamics and retention of metabolites in the intracellular and extracellular compartments, changed the previously established growth characteristics of the cultures, and hampered the total recoveries of arachidonic acid metabolites. In addition, control incubations of the culture media without cells for seven days did not generate any detectable derivative of arachidonic acid in the absence or presence of Wy-14,643. See Results. Thirdly, the levels of 5- and 15-HETE metabolites are also lowered by expression of L-FABP at 24 hours incubation of the transfected cells in serum-free medium (A. W. Panandiker, T. Boyle, M. Jett, and S. Sorof, unpublished results). Fourthly, the strongest support for the present protocol derives from the revelation that L-FABP and Wy-14,643 individually bring about highly specific depressions of cell regulatory and biochemically interrelated eicosanoids in association with their induction of mitogenesis.

The induction of

mitogenesis by L-FABP and the peroxisome proliferator appears to be linked to the depressions of the two triads of cell regulatory and biochemically interrelated eicosanoids. Several implications may derive from these perturbations. Firstly, not to be overlooked is the possibility that the interplay between the two triads themselves and with other cellular constituents may account for the residual interactive effects. Separately, L-FABP expression significantly lowered the levels of extracellular LXA_4 and intracellular LTC_4 and LTB_4 in the presence of Wy-14,643. Noteworthy, extracellular LXA_4 interacts with specific G protein-coupled receptors on cell surfaces (reviewed 31). LTC_4 is an activator of protein kinase C_γ, which is present primarily in brain (32). Protein kinase C is the receptor of the phorbol ester tumor promoters (33, 34), and is involved in the regulation of cell proliferation (reviewed 35-37). Nonetheless, using the present protocol we were unable to detect activation of protein kinase C by LTC_4, LTB_4, or LTD_4 *in vitro* in cytosols of L-FABP expressing cells incubated with Wy-14,643 (10^{-7} M) in 1% calf serum using the present 7-day protocol. In contrast, in another study that employed the transfected hepatoma cells incubated for 48 hours in serum-free medium, L-FABP expression increased multifold factors the ability to activate cytosolic PKC *in vitro* (A. W. Panandiker, T. Boyle, M. Jett, and S. Sorof, unpublished results). Thirdly, Wy-14,643 significantly depressed the intracellular levels of the three L-FABP ligands, 5-HPETE, 5-HETE apparently transitionally, and 12-HETE, in L-FABP expressing cells. A sizable literature exists regarding the growth regulatory activities of hydroxy and hydroperoxy metabolites of arachidonic acid and linoleic acid in cells (38, 39, reviewed 8). Especially noteworthy is the fact that 12(S)-HETE is a critical intracellular signaling molecule that induces activation of protein kinase C and mediates the biological activities of growth factors and cytokines, such as bFGF, PDGF, EGF, and autocrine motility factor (reviewed 39). Furthermore, involvements of arachidonic acid metabolites have also been documented in signal transduction mechanisms of the polypeptide growth regulators, TNF (40), IFN-α (41), and TGF-β (42). Therefore, the combination of that literature and the present findings permit the speculation that our demonstrated depressions of the specific eicosanoids may relieve inhibitions of stimulatory regulators of cell growth. In agreement, the growth inhibitory activity of recombinant bovine MDGI, a homologue of H-FABP, is reciprocally overcome by the growth stimulatory activity of EGF (21). An attractive strategy at this point would be to examine the effects brought about by the depressions of specific eicosanoids on the activities of polypeptide growth factors.

Knowledge that specific eicosanoids are depressed advances the search for the mechanism of the induction of the mitogenesis by L-FABP expression and Wy-14,643. Solution to that problem previously required in principle multiple empirical investigations into the potential involvements of the many possibly relevant ligands of L-FABP. That inductive search is now obviated by a novel rational approach. Various studies now indicate that the cell regulatory activities of L-FABP are modulated by the competitions between the binding of L-FABP with its normal ligands, unsaturated fatty acids and eicosanoids, as opposed to its noncovalent competitive binders, *e.g.*, peroxisome proliferators, and its covalent interactants, *e.g.*, chemically reactive metabolites of the genotoxic liver carcinogens, 2-acetylaminofluorene and aminoazo dyes (reviewed 8). In that context, it is important to note that: (i) the HETEs are regulators of many cellular biological and biochemical activities (38, 39, reviewed in 8); (ii) the HETEs and HPETEs appear to be specifically depressed intracellularly by Wy-14,643 during the induction of mitogenesis by L-FABP and the peroxisome proliferator (this report); (iii) the levels of 5- and 15-HETE metabolites are lowered by expression of L-FABP at 24 hours in serum-free medium, along with multifold elevation of the activation of cytosolic PKC *in vitro* at 48 hours (A. W. Panandiker, T. Boyle, M. Jett, and S. Sorof, unpublished results) during the previously established L-FABP-dependent maintenance of cell morphology and cell survival (13); and (iv) the HETEs and HPETEs were the most avid binders of L-FABP *in vitro* in a previous survey (15). It follows from these associations that the depressions of the two triads of cell regulatory and biochemically related eicosanoids provide a rational basis for the identification of the mediators by which the expression of L-FABP, a target protein of four classes of liver carcinogens, acts with a member of two groups of hepatocarcinogenic peroxisome proliferators in their induction of hepatocyte multiplication.

ACKNOWLEDGMENTS

We thank Eric Ross and Samuel Litwin of the Fox Chase Cancer Center for consultations regarding statistics, members of the Cell Culture Facility of that institution for dedicated cooperation, and Jason E. Foley of the Walter Reed Army Institute of Research for excellent technical assistance in chromatographic separations.

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Table 1. *Concordance of identifications and mass measurements of chromatographically separated eicosanoids as assayed by authentic reference compounds and radioactivity compared to immunofluorescence*

Metabolite	Relative Amount	
	Radioactivity	Immunofluorescence
TxB_2	0.87 ± 0.26	0.85 ± 0.18
LTC_4	0.90 ± 0.12	0.83 ± 0.08
LTE_4	0.83 ± 0.06	0.78 ± 0.05
LTB_4	0.51 ± 0.23	0.53 ± 0.19
LTD_4	1.78 ± 0.33	1.75 ± 0.32
5-HETE	1	1
15-HPETE	1.35 ± 0.20	1.30 ± 0.16
15-HETE	1.61 ± 0.35	1.53 ± 0.31

Metabolites of arachidonic acid were resolved by HPLC and quantified by both radioactivity and immunofluorescence. Amounts were normalized relative to those of 5-HETE. No significant difference was observed in the data obtained by the two methods. The immunofluorescence was specific for the class of compounds. Thus, the LTB_4 antiserum reacted neither with LTC_4, nor HETEs, nor prostanoids. However, LTC_4 antiserum did react with LTD_4, LTE_4, and LTF_4, but not with other metabolites tested. Similarly, the 15-HETE antiserum detected 15-HPETE and 15-HETE-Me, but not 5-HETE, 12-HETE, or other hydroxy derivatives, prostanoids, or leukotrienes. In all cases, the crossreactants eluted in their individual characteristic fractions, and could be quantified separately.

LEGENDS TO FIGURES

Fig. 1. Lowered levels of intracellular and extracellular eicosanoids associated with L-FABP-dependent induction of mitogenesis by the peroxisome proliferator, **Wy-14,643**. L-FABP expressing and nonexpressing transfected hepatoma cells were exposed or not exposed to Wy-14,643 (10^{-7} M) for seven days in 1% calf serum in six experiments of each protocol. The cells and culture media were frozen, defrosted, and the eicosanoids therein were extracted, resolved chromatographically, quantified and identified, as described in the Materials and Methods section. Average contents and standard deviations are shown. The insert contains data with an expanded ordinate.

Fig. 2. Specific depressive effects of L-FABP expression on the intracellular and extracellular levels of eicosanoids in cultures of the L-FABP transfected hepatoma cells. Shown are the statistically significant depressions of levels of eicosanoids of L-FABP expressing *vs.* nonexpressing cells (left panels) and their media (right panels) from cultures treated (upper) or not treated (lower) with Wy-14,643 (10^{-7} M) for seven days. Average contents, standard deviations, and p values of their differences are indicated.

Fig. 3. Specific depressive effects of Wy-14,643 on the intracellular and extracellular eicosanoids of transfected L-FABP expressing hepatoma cells. Shown are the statistically significant depressions in levels of eicosanoids in L-FABP expressing cells (upper) and L-FABP nonexpressing cells (lower) following treatment *vs.* nontreatment of cells (left panels) and culture media (right panels) with Wy-14,643 (10^{-7} M) for seven days. Average contents, standard deviations, and p values of their differences are indicated.

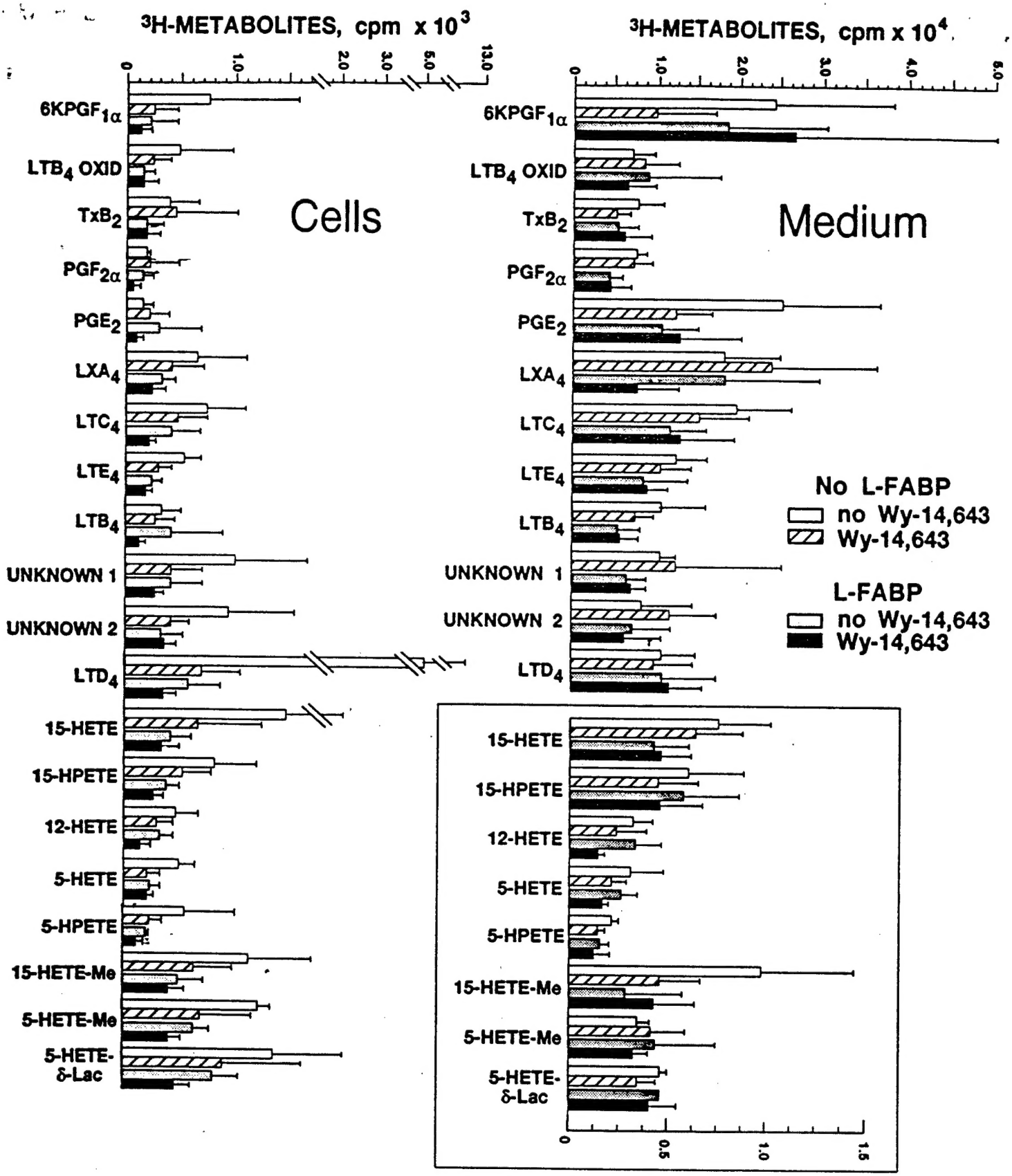

FIGURE 1

Panandiker *et al* "Mitogensis..."

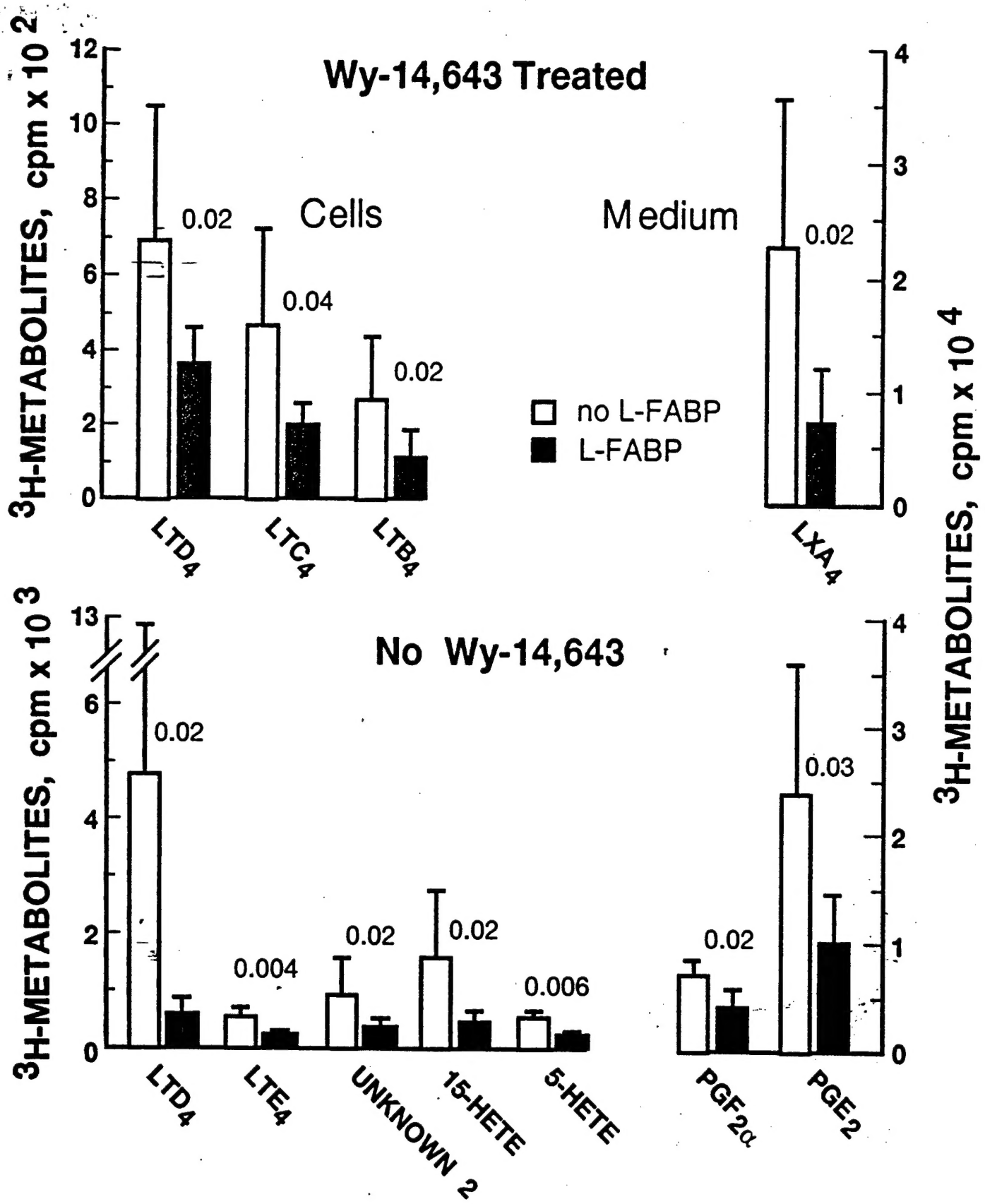

FIGURE 2

Panandiker *et al* "Mitogenesis..."